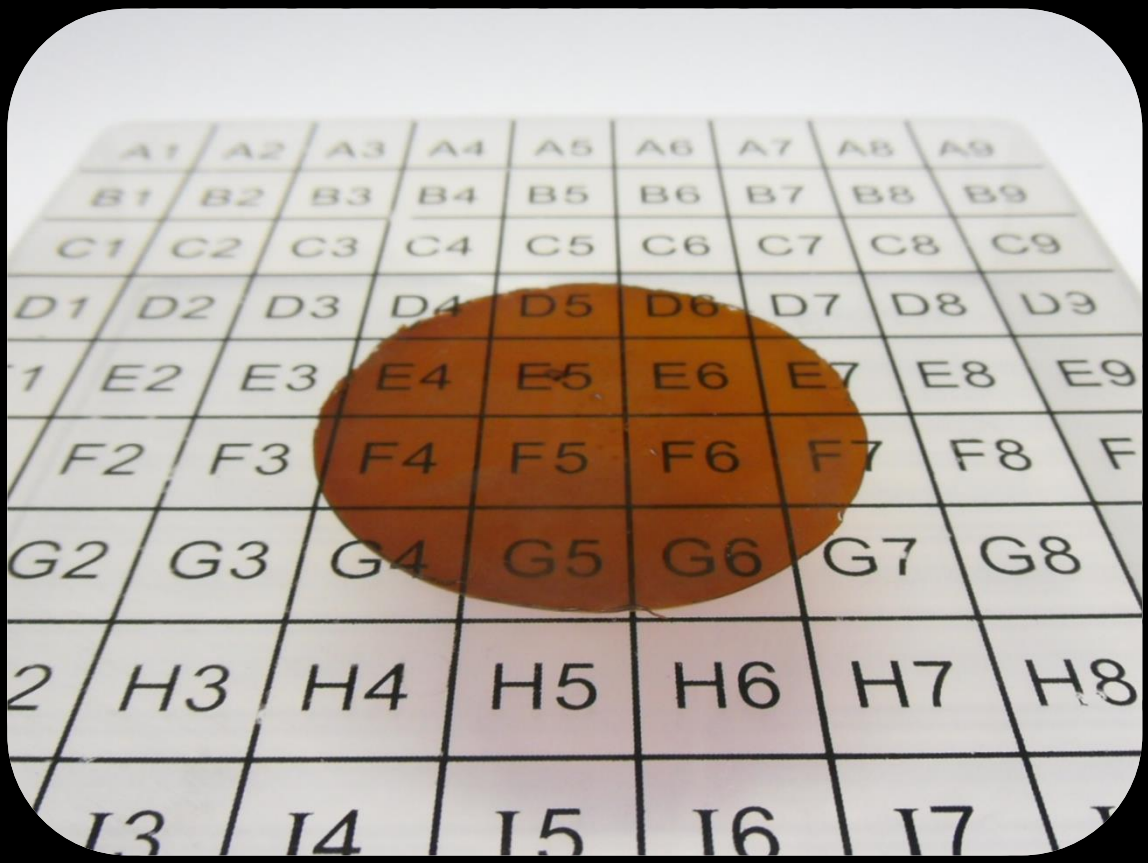
Development of suberin films driven by an ionic liquid-based depolymerisation process

Helga Margarida Correia Ferreira Garcia



Dissertation presented to obtain the Ph.D degree in Chemistry

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras

September, 2013

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA /UNL

Knowledge Creation

Development of suberin films driven by an ionic liquid-based depolymerisation process

Helga Margarida Correia Ferreira Garcia

Dissertation presented to obtain the Ph.D degree in Chemistry

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras, September, 2013

INSTITUTO
DE TECNOLOGIA
QUÍMICA E BIOLÓGICA
/UNL

Knowledge Creation

Title

Development of suberin films driven by an ionic liquid-based depolymerisation process

Cover Image

Suberin film, photo by Rui Ferreira

Second edition

October 2013

Author

Helga Margarida Correia Ferreira Garcia

ISBN: 978-989-20-4163-6

Printed in Portugal

I declare that the work presented in this thesis, except where otherwise stated, is based on my own research. It was supervised by Doctor Cristina Silva Pereira and co-supervised by Professor Luís Paulo Rebelo. The work was mainly performed in the Applied and Environmental Mycology and Molecular Thermodynamics Laboratories of the Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, between January 2009 and June 2013. Part of the results was attained during research visits to the Solution Chemistry Group of the Institute of Physical and Theoretical Chemsitry, University of Regensburg, Germany and to the Macromolecular and Lignocellulosic Materials Group, Department of Chemistry, University of Aveiro.

Financial support for this thesis was provided by Fundação Calouste Gulbenkian for the doctoral fellowship 21-95587-B.

To my family,

Table of Contents

Acknowledgments

In the following lines, I would like to express my gratitude towards all the persons that contributed, directly or indirectly, to this thesis.

My primary appreciation goes to my supervisor Cristina Silva Pereira. I am deeply grateful for the challenges and opportunities you gave me during the last years. Your invaluable guidance, creativity and friendship have been the motor for the development of this work. I am also thankful to Professor Luís Paulo Rebelo, my co-supervisor, for being a source of precious discussions and advices.

I would like to express my great appreciation to Professor Werner Kunz, for welcoming me in his laboratory at Regensburg University, and mostly for his valuable and constructive suggestions during the development of this research work. To Professor Armando Silvestre and his team, who also received me in his laboratory at Aveiro University, and helped me to accomplish important results.

I would also like to thank my thesis committee, Dr. Lígia Martins and Dr. Manolis Matzapetakis, and Dr. Pedro Lamosa, for their availability, critical sense and for the interesting discussions. I am also grateful to Professor Ken Seddon, for all the helpful comments and advices. To Dr. Vitória, for accepting me in her laboratory when I start working at ITQB, and for her kindness and continuous support throughout these years.

I want to thank my former and present colleagues as well as my friends from the Applied and Environmental Laboratory and from the Molecular Thermodynamic Laboratory, for their encouragement, critical sense, break times, and specially for keeping a brilliant atmosphere. A special thank to my "after hours" fellow labmates, partners in "suffering" during our PhDs, for all the support, friendship and fun we shared during the last years. I'll miss you a lot! To my "German family", for making Bavaria a warm place to live. To meet you was extremely grateful, vielen Dank für alles!

I am profoundly indebted for the financial support provided by Fundação Calouste Gulbenkian (doctoral fellowship 21-95587-B). To my host institution, Instituto de Tecnologia Química e Biológica, for the excellent conditions provided to perform this work. A more general thank goes to all the persons enrolled in this institution, for their

constant support, sympathy and for creating an excellent working environment. It was a pleasure to perform my PhD here.

Aos meus amigos, sempre presentes, mesmo que à distância “skype”, pela sua amizade, dedicação, compreensão e sobretudo por vivermos juntos os momentos mais bonitos das nossas vidas.

Above all, I want to express my deepest gratitude to Rui, with whom everything present in this thesis was turned possible. To meet you was undoubtedly the most incredible “discovery” of the last years! I feel very lucky to have you in my life.

Um agradecimento muito especial à minha família, em particular aos meus pais e avós, pelo contínuo apoio, incentivo e educação que me proporcionaram. A vós vos devo tudo o que sou hoje e para vós dedico esta dissertação.

Abstract:

Development of suberin films driven by an ionic liquid-based depolymerisation process

Renewable resources are currently under intense investigation for a variety of different uses, for example as energy sources, for the extraction of value-added chemicals and for the production of novel materials. Nature offers an outstanding diversity of inimitable molecules to serve as starting blocks for the production of such materials. This fact is in alignment with the search for sustainable alternatives to petroleum-based compounds, currently under enormous demand globally.

Suberin, a widely spread cell wall component in the plant kingdom, has stirred high interest due to its heterogeneous chemical composition and its exceptional properties, such as hydrophobicity and high thermal resistance. This complex aromatic-aliphatic cross-linked biopolyester contains a vast and almost unique supply of natural hydroxyacids carrying mid-chain functionalities. Such hydroxyacids constitute excellent building blocks for the production of new biopolymers.

This thesis reports the development of a new suberin material, following the extraction of suberin from cork in an ionic liquid media. Cork, the outer bark of *Quercus suber* L., was selected as the main renewable resource for this project since suberin accounts for half of its dry weight, and a large number of residues generated by the cork stopper industry are readily available. Biocompatible and biodegradable ionic liquids, in particular cholinium hexanoate, were proven for the first time to combine good solvation ability with efficient and selective extraction of suberin (Chapter II and III). Follow-up studies highlighted that the ionic liquid could be used for extracting suberin from other renewable resources (*e.g.* birch outer bark; Chapter IV). In addition, the characterisation of the extracted suberin identified the ability of cholinium hexanoate to combine high extraction yield with partial depolymerisation of suberin (Chapter III). Moreover, the specificity of the ionic liquid was further revealed by analysing the type of bonds cleaved during suberin depolymerisation (Chapter V). The ionic liquid plays the dual roles of a good solvent and of a catalyst, promoting efficient hydrolysis of acylglycerol ester bonds, while keeping the other aliphatic esters partially intact.

Suberin depolymerised from cork by the ionic liquid was comprehensively characterised to identify its chemical and monomeric composition (Chapters III-VI). The selective hydrolysis catalysed by the ionic liquid is the reason why these suberin samples were observed to assemble complex oligomeric and/or polymeric esterified structures, essentially composed of long chain hydroxyacids and ferulic acid monomers. In other words, the suberin cross-linked network was partially conserved in a manner highly likely to resemble that of the *in-situ* suberin (Chapter III and IV). The uniqueness of this macromolecular arrangement facilitated the first production of suberin films (Chapter VI). These were simply cast in water, without any surface modification or purification, following suberin depolymerisation from cork. Key properties of the suberin films obtained were characterised, including hydrophobicity, mechanical and thermal features, as well as, surface morphology. The suberin films showed remarkable antimicrobial properties against both the Gram-positive bacteria *Staphylococcus aureus* and the Gram-negative bacteria *Escherichia coli* (Chapter VI).

In conclusion, the work presented herein constitutes an original approach for the valorisation of suberin from cork. The film forming ability of suberin, at the heart of this work, was observed for the first time in suberin samples recovered following its depolymerisation from cork in an ionic liquid media. The preparation of suberin materials with antimicrobial activity will certainly lead to new applications. This study also furthers the interest in task-design ionic liquids for innovative catalysis.

Resumo

Desenvolvimento de filmes de suberina impulsionado por um processo de despolimerização com líquidos iónicos

Actualmente, os recursos renováveis são amplamente investigados para um vasto leque de utilizações, por exemplo, como fontes de energia, para a extracção de produtos químicos de valor acrescentado bem como para a produção de novos materiais. A Natureza oferece uma excelente diversidade de moléculas inimitáveis que servem como matéria-prima para a produção de tais materiais. Este facto está alinhado com a pesquisa de alternativas sustentáveis para compostos provenientes de combustíveis fósseis, hoje em dia com enorme procura globalmente.

A suberina, um componente da parede celular amplamente difundido no Reino *Plantae*, tem despertado grande interesse devido à sua composição química heterogénea e às suas propriedades excepcionais, tais como hidrofobicidade e elevada resistência térmica. Trata-se de um biopoliéster alifático-aromático complexo e reticulado, maioritariamente composto por hidroxiácidos com funcionalidades epóxi ou diol vicinal no meio da cadeia. Tais hidroxiácidos, extremamente raros na Natureza, constituem monómeros excelentes para a preparação de novos biopolímeros.

Esta dissertação descreve o desenvolvimento de um novo material de suberina, após a sua extracção de cortiça com líquidos iónicos. A cortiça, casca exterior de *Quercus suber* L., foi seleccionada como o principal recurso renovável para este projecto uma vez que a suberina constitui cerca de metade do seu peso, e está disponível em grande quantidade nos resíduos gerados pela indústria da rolha de cortiça. Pela primeira vez líquidos iónicos biocompatíveis e biodegradáveis, em particular o hexanoato de colínium, demostraram ser solventes exímios para a extracção selectiva e eficiente de suberina (Capítulo II e III). Além disso, este líquido iónico também pode ser usado para extrair suberina de outros recursos renováveis (por exemplo, da casca exterior de bétula; Capítulo IV). A caracterização da suberina obtida demonstrou que o hexanoato de colínium extrai suberina com alto rendimento, promovendo apenas a sua despolimerização parcial (Capítulo III). Estudos complementares sobre o tipo de ligações

químicas quebradas durante a despolimerização parcial de suberina, revelaram a especificidade do líquido iónico (Capítulo V). O hexanoato de colínium desempenha um papel duplo de um bom solvente e catalisador, promovendo uma hidrólise eficiente para as ligações éster do tipo acilglicerol, mantendo os outros ésteres alifáticos parcialmente intactos.

A suberina obtida aquando da sua despolimerização da cortiça pelo líquido iónico foi extensivamente caracterizada de modo a identificar a sua composição química e monomérica (Capítulos III-VI). Tornou-se evidente que a hidrólise selectiva catalisada pelo líquido iónico é a razão pela qual as amostras de suberina apresentam estruturas oligoméricas e/ou poliméricas esterificadas, essencialmente compostas por hidroxiácidos de cadeia longa e ácido ferúlico. Por outras palavras, a suberina obtida conserva parcialmente a sua estrutura reticulada original o que leva a crer que esta poderá estar muito próxima da suberina nativa (Capítulos III e IV). A singularidade deste arranjo macromolecular permitiu, pela primeira vez, a preparação de filmes de suberina (Capítulo VI). Estes foram simplesmente preparados por *casting* em água, sem qualquer purificação ou modificação da suberina após a sua despolimerização de cortiça. Algumas propriedades dos filmes de suberina foram analisadas, incluindo a sua hidrofobicidade, as características mecânicas e térmicas, bem como, a morfologia da sua superfície. Os filmes de suberina exibiram propriedades antimicrobianas notáveis contra bactérias Gram-positivas *Staphylococcus aureus* e Gram-negativas *Escherichia coli* (Capítulo VI).

Em conclusão, o trabalho aqui apresentado constitui uma abordagem original para a valorização da suberina de cortiça. A preparação de filmes de suberina foi possível pela primeira vez a partir de amostras recuperadas após despolimerização de suberina de cortiça em líquido iónico. Os materiais de suberina com atividade antimicrobiana, aqui apresentados, certamente levarão ao desenvolvimento de novas aplicações. Este estudo reforça também o interesse na possível utilização de líquidos iónicos para processos catalíticos inovadores.

Members of the Jury

PhD Supervisors

Dr. Cristina Maria da Costa Silva Pereira, Auxiliar Investigator at Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Portugal (supervisor).

Dr. Luís Paulo da Silva Nieto Marques Rebelo, Cathedratic Professor at Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Portugal (co-supervisor).

PhD Examiners

Dr. Alessandro Gandini, Professor at Instituto de Química, Universidade de São Paulo, Brasil.

Dr. José Afonso Rodrigues Graça, Auxiliar Professor with Aggregation at Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Portugal.

Dr. Jorge Fernando Jordão Coelho, Auxiliar Professor at Departamento de Engenharia Química, Universidade de Coimbra, Portugal.

Thesis publications

H. Garcia, R. Ferreira, M. Petkovic, J. L. Ferguson, M. C. Leitão, H. Q. Nimal Gunaratne, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Dissolution of cork biopolymers by biocompatible ionic liquids, *Green Chem.*, 2010, **12**, 367 – 369. (Selected for Green Chemistry cover - Volume 12 Number 3)

H. Garcia, R. Ferreira, A. F. Sousa, M. Petkovic, P. Lamosa, C. S. R. Freire, A. J. D. Silvestre, L. P. N. Rebelo, and C. Silva Pereira, Suberin isolation process from cork using ionic liquids: Characterisation of ensuing products, *New J. Chem.*, 2012, **36**, 2014–2024.

H. Garcia, R. Ferreira, A. F. Sousa, C. S. R. Freire, A. J. D. Silvestre, L. P. N. Rebelo and C. Silva Pereira, Isolation of suberin from birch outer bark and cork using ionic liquids: A new source of macromonomers, *Ind. Crops Prod.*, 2013, **44**, 520–527.

R. Ferreira, **H. Garcia**, A. F. Sousa, M. Guerreiro, F. J. S. Duarte, C. S. R. Freire, M. J. Calhorda, A. J. D. Silvestre, W. Kunz, L. P. N. Rebelo and C. Silva Pereira, Unveiling the dual role of the cholinium hexanoate ionic liquid as solvent and catalyst in suberin depolymerisation, *submitted manuscript*, 2013.

H. Garcia, R. Ferreira, C. Martins, A. F. Sousa, C. S. R. Freire, A. J. D. Silvestre, W. Kunz, L. P. N. Rebelo and C. Silva Pereira, Biomimetic suberin as novel hydrophobic antimicrobial materials, *submitted manuscript*, 2013.

Other publications

M. B. Carvalho, I. Martins, M. C. Leitão, **H. Garcia**, C. Rodrigues, V. S. Romão, I. McLellan, A. Hursthouse and C. Silva Pereira, Screening pentachlorophenol degradation ability by environmental fungal strains belonging to the phyla Ascomycota and Zygomycota, *J. Ind. Microbiol. Biotechnol.*, 2009, **10**, 1249–1256.

M. Petkovic, J. L. Ferguson, A. Bohn, J. R. Trindade, I. Martins, M. C. Leitão, M. B. Carvalho, C. Rodrigues, **H. Garcia**, R. Ferreira, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Exploring fungal activity in the presence of ionic liquids, *Green Chem.*, 2009, 11, 889–894.

M. Petkovic, J. Ferguson, A. Bohn, J. R. Trindade, I. Martins, C. Leitão, M. B. Carvalho, C. Rodrigues, **H. Garcia**, R. Ferreira, K. R. Seddon, L. P. N. Rebelo, and C. Silva Pereira; On the Merge of Fungal Activity with Ionic Liquids towards the Development of New Biotechnological Processes. Ionic Liquid Applications: Pharmaceuticals, Therapeutics, and Biotechnology; 2010, 197-207, *American Chemical Society* (ISBN13: 9780841225473, DOI: 10.1021/bk-2010-1038.ch016).

C. Silva Pereira, R. Ferreira, **H. Garcia** and M. Petkovic, Ionic Liquids, Biocompatibility, Encyclopedia of Applied Electrochemistry, *Springer*, 2012.

C. M. B. Gonçalves, L. C. Tomé, **H. Garcia**, L. Brandão, A. M. Mendes and I. M. Marrucho, Effect of natural and synthetic antioxidants incorporation on the gas permeation properties of poly(lactic acid) films, *J. Food Eng.*, 2013, **116**, 562–571.

L. M. T. Frija, **H. Garcia**, C. Rodrigues, I. Martins, N. C. Candeias, V. André, M. T. Duarte and C. Silva Pereira, Short synthesis of the natural product 3β-hydroxy-labd-8(17)-en-15-oic acid via microbial transformation of labdanolic acid, *Phytochem. Lett.*, 2013, **6**, 165–169.

L. C. Tomé, D. J. S. Patinha, R. Ferreira, **H. Garcia**, C. Silva Pereira, C. S. R. Freire, L. P. N. Rebelo, I. M. Marrucho, Cholinium-based supported ionic liquid membranes: a sustainable route for CO_2 separation, *ChemSusChem*, 2013, in press (DOI number: 10.1002/cssc.201300613).

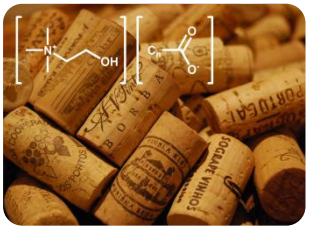
R. Ferreira, **H. Garcia**, A. F. Sousa, C. S. R. Freire, A. J. D. Silvestre, W. Kunz, L. P. N. Rebelo and C. Silva Pereira, Microwave Assisted Extraction of betulin from birch outer bark, *RSC Adv.*, 2013, in press (DOI: 10.1039/C3RA43868F).

Thesis Layout

Chapter I - Introduction

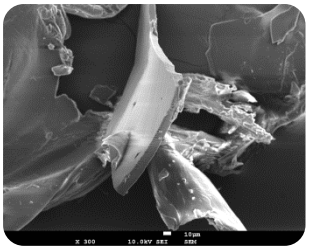
This thesis focuses the development of new biomaterials from plant renewable resources, using alternative depolymerisation methodologies. An overview of the concepts which fall within the scope of this thesis is herein presented.

Chapter II - Dissolution of cork biopolymers in biocompatible ionic liquids



Biocompatible and biodegradable ionic liquids, namely cholinium hexanoate, were proven for the first time to combine good solvation ability with efficient and selective extraction of suberin from cork.

Chapter III - Suberin isolation from cork using ionic liquids: characterisation of ensuing products

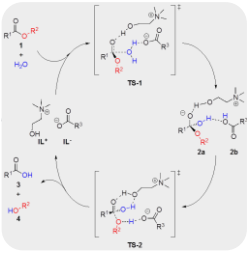


Cholinium hexanoate combines high extraction efficiency with partial depolymerisation of suberin. Suberin obtained through this method shows an esterified and cross-linked nature.

Chapter IV - Isolation of suberin from birch outer bark and cork using ionic liquids

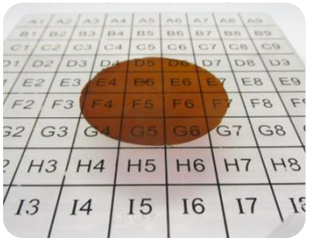
Follow-up studies highlight the ability of the ionic liquid to extract suberin from other sources. Herein, is presented a comparative analysis of suberin samples obtained from cork and birch outer bark, upon extraction with cholinium hexanoate.

Chapter V - Unveiling the dual role of cholinium hexanoate as solvent and catalyst in suberin depolymerisation



The ionic liquid acts simultaneously as solvent and selective catalyst, promoting efficient hydrolysis of acylglycerol ester bonds, while keeping the other aliphatic esters partially intact.

Chapter VI - Biomimetic suberin as novel hydrophobic antimicrobial materials



The uniqueness of the suberin macromolecular arrangement, allows, for the first time, the production of suberin films. These films are moderately hydrophobic, water-proof and show antimicrobial and anti-biofouling properties.

Chapter VII - Concluding remarks & Thesis impact

A critical evaluation of the work herein performed highlighting the strengths, weaknesses, opportunities and threats (SWOT analysis) of this scientific contribution is presented.

Chapter I

Introduction

The subsection “3. Ionic liquids” contains parts of the following author’s publication: H. Garcia, R. Ferreira, M. Petkovic and C. Silva Pereira; Ionic Liquids: Biocompatibility; Encyclopedia of Applied Electrochemistry, *Springer* (available online)

This chapter contains essential information for understanding the subsequent chapters enclosed in this thesis. It does not aim to provide, however, a comprehensive revision of all the subjects herein presented.

1. The context

The initial, although rudimentary, use of natural products, such as starch, cotton, proteins and wool is as old as the beginning of the Human Era. Later with the industrial revolution, XVIII-XIX century, numerous chemical compounds from fossil resources start being used to develop new materials. This period constitutes an important landmark in Human History, when the transition from natural to synthetic products took place. A large number of versatile polymeric materials have emerged, exhibiting a wide range of properties from durability to high thermal resistance or electrical conductivity. This was the beginning of the so-called "plastic age" (Figure 1).[1] Many remarkable examples could be cited at this point, such as the development of Bakelite or Nylon. The first, developed by Leo Baekeland in 1907[1], is a thermosetting phenol formaldehyde resin which has been recently recognised by the American Chemical Society as the world's first synthetic plastic. The second is a thermoplastic polymer, a polyamide, resulted from the extraordinary work of Carothers at DuPont during the 1930s and is still one of the most used polymers nowadays.[2] The chemical progresses made at the time, allowed not only the development of synthetic polymers based on monomers from fossil resources but also the application of new synthetic routes to overcome the limited properties of some already existing bio-based commodities.[2] A notable example was the vulcanisation of natural rubber, which improves its mechanical properties making it useful for tire applications. Nevertheless, the significance of using monomers from renewable resources was neglected, mostly due to the huge quantities of chemicals from fossil resources readily available at low prices.[1,2] Given these circumstances, great effort and investment was placed on the development and improvement of synthetic polymers based on fossil resources. As a result, a myriad of useful materials, such as plastics, inks, textiles, soaps and packaging materials was created. Even after more than one century since the beginning of the "plastic age", these materials remain of utmost importance both in our daily lives and in more sophisticated applications. In fact, the development of new functional materials still remains one of the most active areas of research.

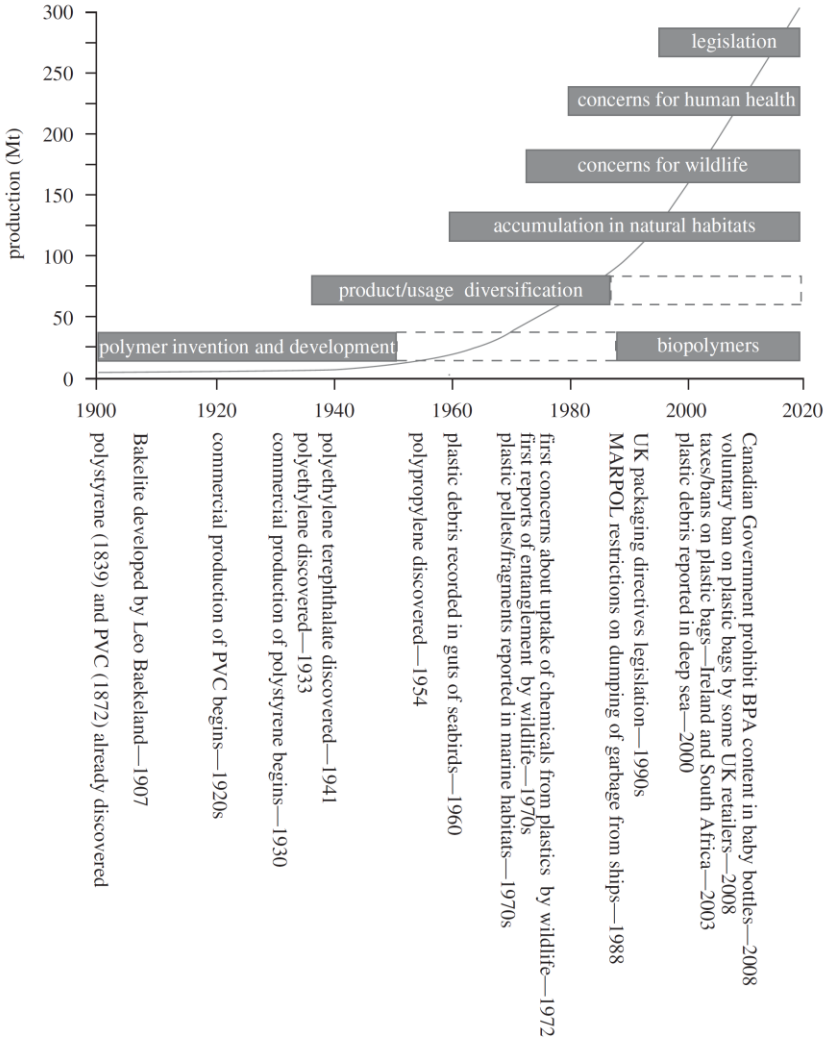


Figure 1| Schematic representation of the historical stages of the development, production and concerns regarding the use of plastics. The line in the graph represents the plastic production in millions of tonnes (Mt). BPA, Bisphenol A; PVC, polyvinyl chloride. (Reprinted from APME (2006)[3])

Throughout the 20th century, along with the intensive exploitation of fossil resources, we became aware of their declining. The fact that these sources are non-renewable within an appropriate period of time, have a serious impact in the world's global consumption of energy, fuel, chemical compounds and synthetic polymers.[4-6] Accordingly, the world population has been facing the raise of geopolitical tensions,

especially denoted by the military conflicts and the increasing fuel prices.[2,6] In addition, the broad use of such feedstocks is at the heart of several environmental problems. On the one hand, the combustion of fossil fuels and related technologies lead to water and air pollution, especially associated to greenhouse gases emissions.[4] On the other hand, synthetic materials are excessively used in short time applications. The lack of biodegradability of such materials, in particular of plastics, leads to their accumulation in different environmental compartments causing serious problems to the ecosystem.[1,7] Suitable alternatives are needed to overcome the high demand of fossil fuels and the pollution problems emerging from their use.

In this context, governments and both scientific and industrial communities have been intensively searching for alternatives that allow sustainable economic growth, *i.e.* the shift from non-renewable hydrocarbons to bio-based feedstocks without generating environmental imbalances or competing with food supplies. The renewed interest in the plant, animal and microbial resources, *viz.* biomass, is being explored in the so-called biorefinery concept; the conversion of biomass to fuels, power, heat and/or chemicals.[6] While for energy there are a number of alternatives, such as solar, eolic and nuclear energies, the production of chemicals strongly depends on the use of renewable resources. In this context, plant biomass such as agricultural and forestry residues constitutes a sustainable and foreseeable source of chemicals. It accounts for a vast amount of abundant biopolymers, such as cellulose, hemicelluloses, starch and suberin (in the heart of this thesis) that might be converted in valuable chemicals or energy.[2] Many challenges are envisaged here in order to obtain sustainable biorefinary platforms, such as overcoming plant biomass recalcitrance and, the development of “greener” and cost efficient separation technologies.[8,9]

The present dissertation aims to contribute to the development of new biomaterials, from plant renewable resources, using alternative extraction methodologies. Hence, the following lines within this chapter focus on particular features regarding the state of the art of the selected biopolymer, suberin, and on ionic liquids, the alternative solvents used to depolymerise suberin from its natural sources.

2. Suberin

Why is suberin worth to study?

Suberin is a natural polyester widely present in the plant Kingdom. It is deposited in the secondary cell wall of specialised tissues, particularly in the phellem of tree barks and in some subterranean parts,[10] during the plant development or as a response to external stress and wounding.[11,12] It builds a hydrophobic barrier that regulates the loss of water, nutrients and gases hampering the entrance of pathogens and also acting as a filter towards the injurious solar radiation.[12-14] These physiological functions arise most certainly from the macromolecular assembly of suberin monomers in a complex matrix embedded with other cell wall components (lignin, polysaccharides and extractives).[13] This structural organisation, yet to be comprehensively understood, constitutes presently an important knowledge gap. Moreover, the occurrence of suberin in different plant tissues and species makes its study even more challenging[14].

Despite still under debate, it is generally accepted that suberin comprises an aromatic and an aliphatic domain, which are structurally organised in a complex cross-link network. [10,15,16] Scientific evidences show that suberin holds high abundance of α,ω-alkanedioic acids and ω-hydroxyalkanoic acids and their corresponding derivatives carrying *mid*-chain functionalities such as unsaturated, epoxy or *vic*-diol groups.[2,12] This singular molecular diversity, barely found in other sources, fosters high interest for suberin exploitation as a source of monomers for the production of new polymeric materials.[16]

Although present in a vast spectrum of plants, suberin is particularly abundant in the outer barks of cork oak and birch as well as in the peel of potato species, *Quercus suber* L., *Betula pendula* R. and *Solanum tuberosum* L., respectively.[2] Accordingly, these species have represented important models for the study of suberin during the last decades.[10,16] Moreover, the outer bark of *Quercus suber* L. and *Betula pendula* R. constitute economically important raw materials for the cork manufacturing and for the paper pulping industries, respectively.[2,17] Both industries generate, however, huge

amounts of by-products *per* year. During cork processing *ca.* 25 wt% of the total production is discarded in the form of residues (*ca.* 50 000 ton/year in Portugal).[18] In the paper industries birch logs are debarked prior to pulping. In a facility that processes *ca.* 400 000 tonnes of birch *per* year, the outer bark residues correspond to *ca.* 20 000 tonnes.[19,20] Despite the high content of suberin (*ca*. half of their weight) both residues are simply burned to produce energy[21]. Similarly, *Solanum tuberosum* L. species are also industrially exploited mainly for food and feed purposes. The peeling of potatoes results in losses that range from 15-40 wt% of their initial weight depending on the procedure applied, *i.e.* abrasion, steam or lye peeling.[22] This fraction, with no significant commercial value, represents a serious disposal problem to industry.[23] The valorisation of all the above mentioned suberin residues have been already envisaged[16,24-27], though only modestly exploited so far.

Given the high amounts of suberin enrich residues and the inherent value of its composing monomers, suberin seems to be an excellent source of inspiration for the development of new biomaterials aiming the preservation of its physical, chemical and biological properties.

2.1 Depolymerisation methods

The analysis of suberin monomeric composition is not possible without hampering its *in-situ* structure, *i.e.* without using depolymerisation procedures to isolate suberin from the plant cell wall matrix.[10,12] In addition, prior to suberin extraction, and depending on the suberin source, pre-treatments are usually required. For instance, Soxhlet extraction[28] and/or enzymatic treatments[29] are normally used to remove the extractable non-covalently bonded compounds and the polysaccharides, respectively[12].

As aforementioned suberin is a biopolyester, therefore its depolymerisation is attained with treatments involving ester bond cleavage.[16] The first studies concerning the monomeric composition of suberin, and of structurally related biopolymers, have been reported during the last century ([30-33] and references therein). Although, only with the evolution of the gas-chromatography coupled to mass spectrometry (GC-MS)

analysis the accurate suberin monomers identification, without ambiguity, could be obtained. This tandem technique is still of great importance today.

Several suberin depolymerisation methods have been implemented, namely alkaline hydrolysis (saponification), alcoholysis (transesterification) and hydrogenolysis (reductive cleavage).[16,31,34] These methodologies led mainly to the collection of aliphatic suberin extracts described as showing a viscous/pasty consistency[16]. The depolymerised suberin samples are greatly affected by the method applied.[35] For instance, variations can be observed regarding suberin extraction yields and suberin composition due to preferential removal of specific monomers and/or induced chemical modifications in some functional groups, such as in the epoxy moieties[36]. Hence, the depolymerisation method should be carefully selected depending on the ultimate goal, *i.e.* suberin detailed chemical composition, monomers isolation or the study of suberin structure.

Alkaline hydrolysis

The alkaline hydrolysis consists in a saponification reaction, where the ester groups are converted into the corresponding carboxylate salts and alcohols. The carboxylic acids are afterwards regenerated through acidification and subsequently extracted with organic solvents. This reaction can be applied using potassium hydroxide[30,36] either in water or in aqueous alcoholic solutions leading to an extensive cleavage of ester bonds with the conversion of the epoxy rings into *vic*-diol groups. In general, alkaline hydrolysis reactions are performed with harsh conditions (*e.g.* 2M of potassium hydroxide[35]), which may provoke major degradation of the monomers.[33,35] However, *Ekman et al.* demonstrated that the epoxy groups can be virtually preserved in an alkaline hydrolysis when using potassium hydroxide in a mixture of ethanol with only vestigial amounts of water.[20] Interestingly, the alkaline hydrolysis of wound-healing potato tissues revealed the existence of feruloyltyramine and feruloyloctopamine dimeric structures.[37] This fact constituted an important evidence, showing that suberin aromatic monomers can be linked through non-ester bonds.

Alcoholysis

Alcoholysis approaches consist of a transesterification reaction, which is normally attained with methanol (as the alcohol reagent) and sodium methoxide (as the strong catalytic base), under solvent reflux conditions. The alkaline methanolysis constitute the most commonly used suberin depolymerisation method, allowing the complete removal of suberin monomers in the form of methyl esters (*e.g.* using *ca.* 0.05 to 3% of sodium methoxide refluxing in methanol from 2 to 3 hours[28,33,38]). This approach leads to the conversion of the epoxy ring into a methoxyhydrin, which due to its absent in native suberin constituted a major evidence of the presence and position of epoxy moieties in suberin samples.[33]

This method has been also carried out with low concentration of sodium methoxide[38], as well as with different catalysts, such as calcium hydroxide[39-42] or calcium oxide[34,43,44]. The first alteration allows the partial conservation of the epoxy moieties, whereas the second leads to a mild alkaline methanolysis, though with a much lower depolymerisation yield. Importantly, these mild methanolysis conditions have contributed for important insights into the *in-situ* structure of suberin (see section Glycerol for further details). Another advantage of such adjustable conditions is the selective solubilisation of specific groups of monomers, as reported by Lopes *et al.*[28].

Methanolysis catalysed by acids is also possible (*e.g.* using BF_3[32,45], HCl[35] or H_2SO_4[36]), although with a slower reaction rate when compared to the alkaline hydrolysis. Such depolymerisation conditions led to the opening of the epoxy rings.[36]

Hydrogenolysis

Depolymerisation of suberin through hydrogenolysis with lithium aluminium hydride ($LiAlH_4$) in tetrahydrofuran, has also been reported.[12,32,45] The ensuing products of such reaction are composed solely of alcohols, due to the extensive ester reduction. Accordingly, epoxy rings are also converted into *vic*-diols. The use of labelled $LiAlD_4$ allowed remarkable advances in structural studies as it introduces deuterium in the moieties where the reduction takes place.[12,31,45]

Other methodologies

Other methods and characterisation techniques were also implemented to evaluate the chemical composition of suberin samples (*e.g.* flash pyrolysis-GC-MS[46]). Evidences that suberin comprise aliphatic[47] and aromatic moieties[12] were also attained with histochemical stainnings.[48] In addition, to gain further insights into suberin aromatic composition, typical degradative techniques used for lignin were also applied to suberised tissues, such as alkaline nitrobenzene oxidation[31] and thioacidolysis[49].

The use of non-degradative spectroscopic techniques, such as Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR, solid and liquid state), showed to be appropriate to further characterise suberin.[13,50,51] In particular, relaxation experiments in solid state NMR revealed different motions in methylenic chains, which have contributed to important structural information, both on suberin from cork[28] and from potato tissues[52]. Another example of success was the use of labelled precursors to follow the molecular intermediates of suberin biosynthesis in potato wound periderm by solid state NMR[53]. Such approach suggests the occurrence of phenolic amides in suberised potato tissues as a primary response to wounding[37] and provided evidences for their extensive cross-linking[54].

2.2 Monomeric and oligomeric composition

Suberin content and monomeric composition is highly variable, and depends on the depolymerisation and analytical methodologies used, as well as on suberin natural source.[2,10,35] Detailed monomeric composition has been mostly attained by GC-MS. Although care should be taken when comparing results from different reports, there are some general aspects to be considered. In order to obtain an accurate quantification, the GC-MS analyses of suberin mixtures should take into account the use of internal standards and their chromatographic response factors.[10,16,28,38] The monomeric mixtures are injected after a derivatisation, normally attained by trimethylsilylation (TMS), which facilitate the volatisation of the compounds allowing their detection in the form of trimethylsilyl derivatives. The following lines present an overview of common monomers and oligomers detected in suberin, which are also represented in Figure 2.

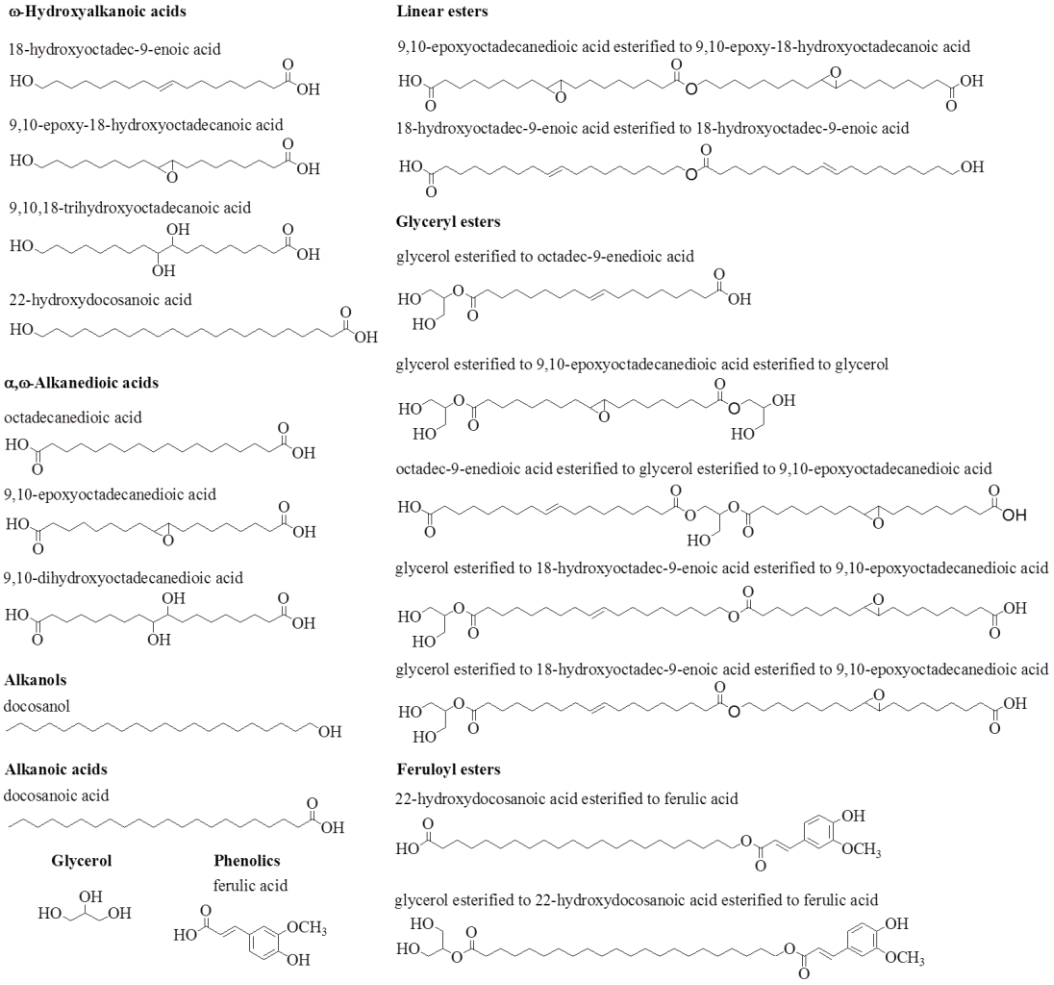


Figure 2| Representative chemical structures of monomers and oligomers detected after suberin depolymerisation.

Aliphatic monomers

The aliphatic monomeric components present in suberin are mainly ω-hydroxyalkanoic acids and α,ω-alkanedioic acids, followed by minor amounts of alkanoic acids and alkanols. These monomeric families are essentially composed of even-numbered chains ranging from C16 to C26, although for the case of alkanoic acids the presence of saturated C12 and C28-30 and *mid*-chain derivatives of C18 were also sporadically found. ([16] and references therein) The C18 and C22 ω-hydroxyalkanoic acids and α,ω-alkanedioic acids represent the most abundant suberin monomers. A significant fraction

of these acids contain *mid*-chain unsaturations, epoxy rings or *vic*-diol groups. The relative abundance of such monomers differs from species to species. For instance[2], in the case of cork suberin, the most abundant monomers are 9,10-epoxyoctadecanedioic acid (38%), 22-hydroxydocosanoic acid (*ca.* 29%) and 18-hydroxy-9-octadecenoic acid (*ca.* 18%). In contrast, the main monomers found for suberin from birch outer bark are the 9,10-epoxy-18-hydroxyoctadecanoic acid (*ca.* 39%), followed by 22-hydroxydocosanoic acid (*ca.* 14%) and 18-hydroxy-9-octadecenoic acid (*ca.* 12%).

Aromatic monomers

Ferulic acid is undoubtedly the most frequently found aromatic monomer in suberin, along with smaller amounts of other structures such as vanillin, benzoic acid derivatives and caffeic acid. ([16,55] and references therein) As aforementioned, depending on the depolymerisation methods used, aromatic dimeric structures[37] (feruloyltyramine and feruloyloctopamine) as well as feruloyl esters[55] (such as hydroxycinnamates of ω-hydroxyalkanoic acids[56] or hydroxycinnamates of alkanols[44,57]) could also be found. In addition, vestigial amounts of monolignols were also found, such as *p*-coumaroyl, coniferyl and sinapyl alcohols. ([16,55] and references therein)

Glycerol and suberin oligomers

Glycerol has been recognised, for a long time, as a suberin component and therefore quantified in many studies ([10] and references therein). Although only in the past decades, the importance of glycerol has been fairly recognised as a key cross-linking monomer of the acid features. The partial depolymerisation of suberin, under mild methanolysis conditions, led to the release of monomeric and oligomeric structures. In addition to the GC-MS detection approaches[38,43], solubilised structures were also analysed by electospray ionisation coupled to tandem mass spectrometry (ESI-MS/MS) since it allows the detection of compounds with higher molecular weights[41]. These studies reported, for the first time, the existence in suberin of glyceryl esters[41-44,57], feruloyl esters[39,44,57] and more recently the presence of linear dimeric esters[42] either between ω-hydroxyalkanoic acids and α,ω-alkanedioic acids, either with two ω-hydroxyalkanoic acids.

"Mind the gap"

Another important fact is the uncompleted GC-MS quantification of suberin samples. The identified amount of suberin monomers is highly variable and, reported to be 27-74 % for the case of *Quercus suber* L. cork[17,28,30,38,58], *ca.* 60-70 % for *Betula pendula* R.[17,30] and *ca.* 60 % potato periderm[38,57]. The remaining part of the depolymerised suberin is reported to be oligomeric or composed of high molecular weight fragments, consequently non-volatile and therefore non-detectable in GC-MS ([16,58]). The latter has been associated with suberan, an insoluble, non-hydrolysable aliphatic suberin-like biopolymer located in the periderm or endoderm of plants.[59,60] Recent studies suggested that the polymethylenic structures of suberan present a high degree of crystallinity, which allow its distinction from suberin.[60]

2.3 Monomer assembly and in-situ *structure*

Since the early compositional descriptions of suberin, even before the 1970s ([30,31] and references therein), huge efforts have been done to reveal its *in-situ* macromolecular structure. As mentioned above, suberin has been studied mainly in potato periderms and in the outer bark of several trees, though limited to the depolymerisation methods and the characterisation techniques used so far. These studies allowed the disclosure of suberin monomeric compounds and also to shed light on the intra and inter molecular interactions between suberin and the other cell wall components.

It has long been known that suberin is a recalcitrant biopolyester composed of aromatic and aliphatic monomeric units.[31,45,61] Since then, several suberin models have been proposed to illustrate its *in-situ* structure. Despite the actual discussion, currently the prevailing idea is that suberin comprises two covalently cross-linked domains, the major being the polyaliphatic domain and the minor the polyaromatic one.[10,15,16,34,62]

The polyaromatic domain structure and composition is not yet fully understood, especially due to its recalcitrance and chemical similarity with lignin, consequently difficult to distinguish.[54,63] Even so, solid state NMR studies and chemical analyses show that these domain is ingrained on the inner face of the

primary cell wall[10,15,16,34,52,64] and composed of hydroxycinnamic acids and its derivatives and vestigial amounts of monolignols. Evidences support the idea that these compounds are extensively cross-linked between each other and to other cell wall compounds *via* stable carbon-carbon, amide and ether bonds.[16,49,54,55] In addition, the same studies revealed the presence of a second spatially distinct aromatic population within the polyaliphatic domain (see below).

The polyaliphatic domain is mainly composed of ω-hydroxyalkanoic acids and α,ω-alkanedioic acids and the corresponding *mid*-chain unsaturated, epoxy or *vic*-diol derivatives (see section 1.2.1 for detailed aliphatic monomeric composition). In addition, the deposition of hydroxycinnamates (predominantly ferulic acid) leads to the typical lamellar organisation of alternate aliphatic and aromatic components.[15] These monomers are aligned and linked *via* linear aliphatic ester or acylglycerol ester bonds. The presence of glycerol is particularly important, as it acts as a key cross-linker in the formation of a three-dimensional network, connecting hydrophilic moieties and both suberin domains. [15,55] The distance between two acylglycerols has been associated with the constant thickness of the suberin lamellar arrangement,[15,65] although this structural arrangement is not always observed in the particular case of cork[66].

It is important to reinforce the idea that suberin monomeric composition diverge among different plant species, which will certainly lead to slight modifications in the resulting structure. In other words, the relative abundance of different functional groups contributing to the assembly of suberin is different from species to species. Regardless of this observation, the monomeric assembly will be based in the binding of compounds that are prone to cross-link (≥3 OH or COOH groups) and compounds that are prone to linearly expand the structure (with 2 functional groups). Moreover, most of the monomers present in suberin comprise *mid*-chain functionalities that can promote covalent links between other monomers or further stabilise the structure through H-bond interactions.[10,34]

In 2007, Pereira has proposed a model that considers the current structural findings for cork suberin.[10] This model, reprinted in Figure 3, aims to illustrate a

suberin oligomeric fraction, taking into account the relative abundance of the different monomers. In detail, glycerol is represented as the key cross-linker involved in the following structures: (i) glycerol – α,ω-alkanedioic acid – glycerol; (ii) ω-hydroxyalkanoic acid – α,ω-alkanedioic acid and (iii) glycerol – α,ω-alkanedioic acid – ferulic acid.

So far, however, there is still insufficient knowledge to accurately define the *in-situ* macromolecular suberin structure, the way it connects to the other plant cell wall components and how it is spatially organised.

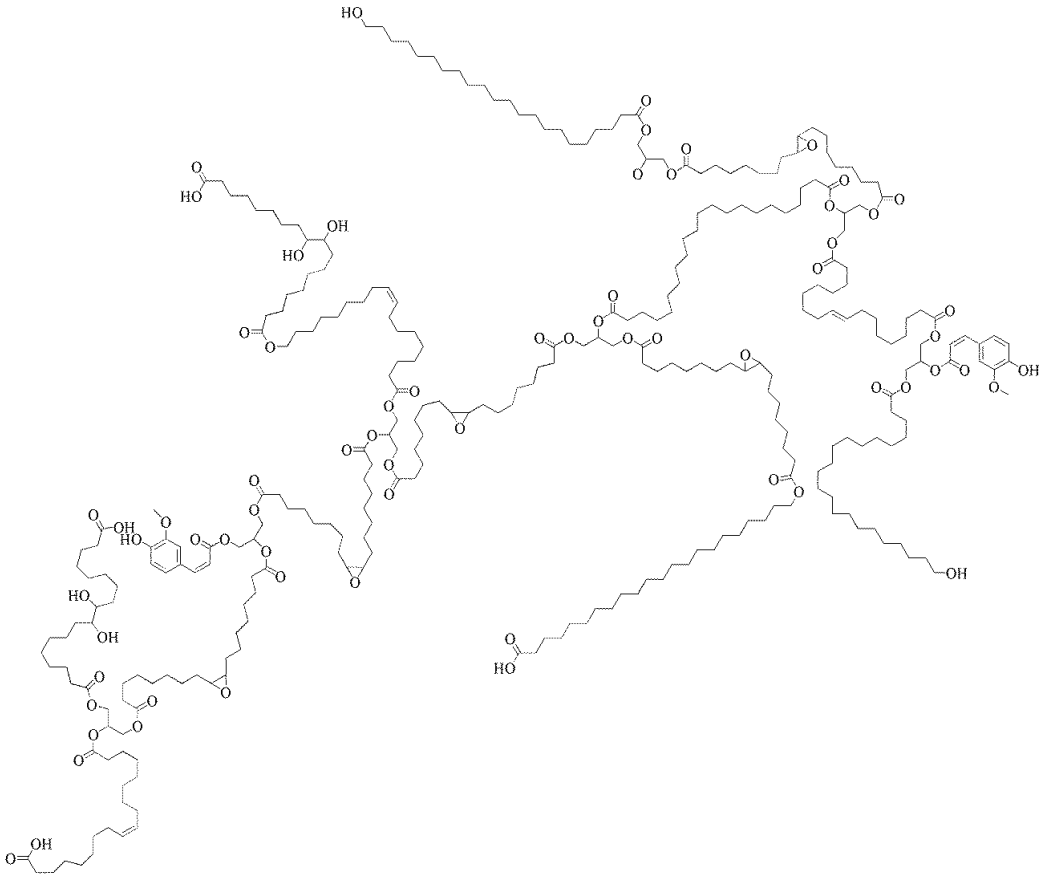


Figure 3| Proposed model for a suberin oligomeric fraction from *Quercus suber* L. cork, adapted from Pereira.[10]

2.4 Applications

The interest on suberin is spread in diverse scientific disciplines, from biology, *e.g.* the understanding of the polymer biosynthesis,[11,67] to chemistry, *e.g.* the depolymerisation and characterisation of suberin[16]. Although only modestly exploited so far, knowledge on suberin physical, chemical and biological properties have inspired ideas for its potential use. On the one hand, suberin monomers and oligomers have been regarded as a source of additives or building blocks for the synthesis of novel macromolecular materials. On the other hand, it has also shown potential for pharmaceutical and cosmetic applications. The purpose of the following lines is to provide information on the few available examples of potential suberin applications.

Some studies reported the use of suberin model monomers and suberin extracts, from birch outer bark and/or cork, as precursors for the synthesis of suberin-based polyurethanes[68,69] and polyesters[24-26,70]. Suberin depolymerisation products, obtained from extensive ester cleavage reactions, were used as additives for printing inks[71] or polymerised either directly[24], mixed with co-monomers[25,69] or after laborious purification of selected monomers[70]. Importantly, efforts were made to produce novel suberin-based polyesters *via* green synthetic pathways using enzymes[24,26,70], microwave[26] or mild polycondensation and transesterification reactions[24-26]. Another significant approach, which has been also successfully applied in other renewable materials[2], is the oxypropylation of suberin enriched residues, namely cork powder, aiming to the preparation of polyurethane foams.[72-74]

Promising results reported that suberin extracts from *Quercus suber* L. presents antimutagenic properties[75], is a carcinogenic absorbent[76] and can also be used as an additive for anti-aging active ingredients[77]. In fact, such suberin extracts are already being commercialised as cosmetics that display an instant smoothing, lifting and tensor effect on skin (*e.g.* Black Swan®, Uplift Skincare® and Suberskin®).

Other interesting suberin applications, though far from being implemented, have also been envisaged concerning the study of its biosynthesis[67]. Some authors have suggested that the future might hold suberin engineering strategies to improve the

resistance of crops towards environmental conditions[78]. Another major challenge would be the biotechnological production of oxygenated fatty acids using the enzymes involved in the suberin biosynthesis.[79]

2.5 Suberin-related biopolyesters

Although not in the scope of this thesis, one cannot disregard the natural occurrence of cutin, an important suberin-related plant biopolyester. In contrast to the suberised tissues, cutin is deposited at the outer surface of the epidermal cell walls, such as leaf and fruits. This cross-linked biopolyester have also been extensively studied through isolation and depolymerisation methods similar to those used for suberin.[12,31] It is physiologically and chemically related to suberin, though essentially composed of C16 and C18 ω-hydroxyalkanoic acids compounds and its *mid*-chain *vic*-diol and epoxy derivatives.[12,14] When compared to suberin, the main compositional differences of cutin are the absence of α,ω-alkanedoic acids and the presence of only vestigial amounts of aromatics. These characteristics contribute to slight variations regarding the structures of both biopolyesters. Glycerol also plays an important role in the cutin structure contributing for the binding of ω-hydroxyalkanoic acids, which can be either linearly connected or cross-linked.[80,81]

Similarly to that abovementioned for suberin, studies involving the synthesis of cutin mimicking polymers using either model hydroxyalkanoic acids or cutin extracts were also performed.[82-84] These cutin biomimetic materials aimed at the creation of biopolymers with enhanced performance[83], and have also given insightful information regarding the still barely studied self-assembly of *in-situ* cutin.[84,85]

In addition, many other natural polyesters could be pointed out here, such as shellac (a resin secreted by *Kerria lacca*)[86,87], vegetable oils[2,88] or poly(hydroxyalkanoates)[89]. The latter, mainly produce by engineered bacteria, have been claimed to be an inexpensive alternative for petroleum-based polymers.[2] These biopolyesters are also somehow suberin related polymers, though with much less complex structures.

3. Ionic liquids

"In the following, I will disclose my investigations on the electric conductivity and, derived from the capillarity constant, the molecular size of some organic ammonium salts. Anhydrous salts were chosen, which melt at relatively low temperatures, approximately up to 100°C. These low melting points limited the degree of thermolysis of both the solvent and the dissolved salts in the molten salt." Paul Walden in 1914[90]

A new class of neoteric solvents emerged in the beginning of the 20th century.[90] However, only in the 1990s with the discovery of air and water stable room-temperature 1-methyl-3-ethylimidazolium salts[91], the (recently coined) field of ionic liquids has gained prominence. Their general definition derived from the aforementioned citation: ionic liquids are salts solely composed of ions which melt at temperatures up to 100°C. At the present (COIL5 2013), the scientific community is arguing that their maximum melting temperature should be neglected as it has no physical or chemical meaning[92].

Perhaps one of the most fascinating features about ionic liquids is their tailored design.[93] Ionic liquids offer the independent selection of their composing ions, allowing the fine tune of their thermophysical, chemical and biological properties to address specific requirements. The spectrum of attainable properties, such as hydrophobicity, low viscosity and high conductivity, is increasing as new ionic liquids are discovered. There are millions of estimated possible ionic liquids formulations, though only *ca.* 300 are commercially available.[92] The most commonly found cations and anions, either organic and/or inorganic, are represented in Figure 4.

Ionic liquids combine the Coulombic environment of inorganic salts with the organic functionalities present in common solvents. When compared with common salts (*e.g.* sodium chloride melts at 801 °C), ionic liquids present considerably lower melting temperatures. This results from the poor packing efficiency of their usually asymmetrical ions and from the delocalisation of charges. In addition, theoretical studies revealed that ionic liquids are nanostructured[94] showing segregated polar and non-polar domains[95]. These findings constituted a major breakthrough towards the understanding of the unique solvation properties of these liquid salts and their interactions with other solutes.[96]

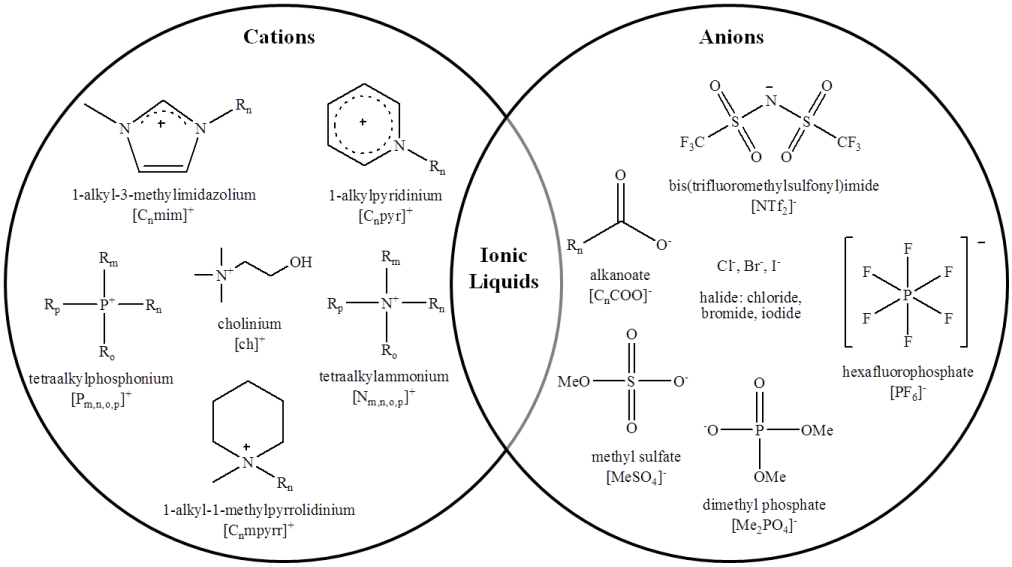


Figure 4| Common cations and anions used in ionic liquids formulations.

The rapid development of the ionic liquids field is mainly related to the aim to reduce or eliminate hazards associated with traditional solvents. Ionic liquids' negligible vapour pressure[97] and non flammability (with some rare exceptions) have led to their classification as "green" and non toxic solvents. Common generalisations that ionic liquids are either "green" or "toxic" solvents should however be avoided: both extremes are totally misleading. Critical aspects of ionic liquids toxicity[98] and biodegradability[99] should be considered for each ionic liquid and in the context of its application.

The most commonly studied ionic liquids family are the imidazolium-based ones. These solvents usually present low melting points and viscosities, high chemical stability and are commercially available at acceptable prices, which make them appealing for broad range applications. However, it is generally accepted that aromatic cations present high toxicity, especially those carrying long alkyl chains.[98] Evidently, when accounting for toxicity and environmental persistence these solvents do not fully address the important principles of green chemistry. This constitutes a good example of the aforesaid, reinforcing the idea that a careful evaluation of the ionic liquid should be taken into account.

The conscious design of ionic liquids (or any solvent) should incorporate not only low toxicity and high degradability, but also the remaining principles of Green Chemistry.[100] In brief, one should also consider the impact of ionic liquids synthesis and application, as well as their lifecycle and the products resulting from their degradation. The understanding of ionic liquids (their core chemistry, synthesis and purification methods) has advanced significantly over the past decade, and is currently set on solid ground, opening doors to the design of biocompatible ionic liquids[101], incorporating (*inter alia*) amino acids[102], carboxylic acids[103-105], non-nutritive sweeteners[106], or glucose[107]. The selection of benign cations, such as cholinium[108], constitutes, still today, one of the most important advances towards their conscious design. The quaternary ammonium salt, cholinium chloride, classified as a provitamin in Europe and widely used as animal feed supplement[109], has been extensively investigated[99,110-112]. Cholinium chloride environmental sustainability is also made known by the synthesis process: a one step solvent-free reaction of hydrogen chloride, ethylene oxide and trimethylamine.

Undoubtedly, ionic liquids constitute a class of versatile solvents that can be tailor-designed in order to fulfil the needs of a specific purpose. This is clearly an advantage when compared to conventional organic solvents. The structure activity relationships constitute essential tools to deliver safer ionic liquid formulations with enhanced technical performance. This class of neoteric solvents are breaking new ground in chemistry allowing their use in a huge number of applications, such as electrochemistry[113], chemical synthesis[114], catalysis[115], separation processes[116], and solvents for dissolution of bio-related materials (*e.g.* biomass[117,118] and proteins[119]). In addition, the aforementioned ionic liquids properties, together with their potential to be recycled and reused, encouraged their application at the industrial level. Several processes have been implemented, at both commercial and pilot scales in companies such as BASF (*e.g.* the BASIL™ process), *Institut Français du Pétrole*, BP, Chevron, PetroChina, amongst others.[92]

3.1 Application in biomass processing

As aforementioned (see section 1.1) lignocellulosic biomass is the most abundant plant resource on Earth and is being intensively investigated for its conversion into biofuels and/or platform chemicals. However, a competitive cost-effective technology for lignocellulosic utilisation is hampered by its recalcitrancy.[8] In this context, ionic liquids offer new possibilities towards biomass dissolution and have been widely investigated. In fact, this is well denoted by the numerous reviews available in recent literature.[117,118,120-122] Ionic liquids allowed the solubilisation and deconstruction of diverse lignocellulosic materials (*e.g.* wood[123-126], swichgrass,[127,128] and straws[124]) and of their components (*e.g.* cellulose[129] or lignin[130]). There are numerous factors affecting their dissolution efficiency (*e.g.* time, temperature, water content, cellulose crystalline degree, natural biomass variability, particle size), yet some common trends are worth to be mentioned.[117,118,120,122]

So far, the most suitable ionic liquids reported for cellulose dissolution comprise basic anions, *i.e.* with hydrogen bonding acceptor ability (as determined by Kamlet-Taft parameters).[117,121] Accordingly, NMR[131] and theoretical simulations[132,133] studies revealed that basic anions strongly interact with the hydroxyl groups present in cellulose, which allow the disruption of its intra and intermolecular hydrogen bond network. On the contrary, the role of the cation is not yet fully understood.[117,121] Even though, formulations combining pyridinium or imidazolium cations with basic anions, such as carboxylates, dialkyl phosphates or halogenates, were reported to be the most efficient and therefore have been widely studied.[117,118,120,122] The dissolution of lignin, however, was more efficient when ionic liquids carrying only moderate basic anions were employed (*e.g.* imidazolium sulphate, acetate or chloride).[117,121] As expectable, the efficient deconstruction of lignocellulosic materials requires ionic liquids with properties similar to those mentioned above.

The water content present in the reaction media is another important factor for the efficient deconstruction of lignocellulosic materials.[117,121] On the one hand, the presence of limited quantities of water promotes the cleavage of lignin ether bonds, avoiding self-condensation reactions of its composing monomers.[121] On the other hand, water can act as

an anti-solvent in the dissolution of cellulose and lignocellulosic materials. A remarkable exception was recently reported by Ohno and co-workers.[134] In this work, cellulose (15 wt%) was rapidly (5 min) dissolved at room temperature in tetrabutylphosphonium and tetrabutylammonium hydroxide aqueous solutions (40-50 wt% of H_2O).

In general, the deconstruction of lignocellulosic materials results in a mixture of their components.[117,121] Hence, different precipitation approaches could be employed depending on the ultimate goal. For instance, some authors proposed that mixtures of organic solvent-water can lead to the preferential cellulose precipitation, whilst lignin remains in solution.[123,126] Moreover, the regenerated cellulose revealed a lower degree of crystallinity when compared to its native state, *i.e.* upon dissolution in the ionic liquid cellulose I is converted into cellulose II.[127] This facilitates either the enzymatic or chemical conversion of cellulose into fermentable sugars for the production of biofuels or valuable chemicals, such as hydroxymethylfurfural.[135]

Many other examples could be cited here, regarding the deconstruction of other types of biomass, such as chitin and chitosan[136], keratin[137,138], starch and zein[139], vegetable oils[140,141] and algae[142]. This field of ionic liquids applications is extremely vast and will not be described in further detail.

4. Concluding remarks

The outstanding solvation ability of ionic liquids, in particular their capacity to dissolve wood[126], prompted us to develop a new methodology to extract suberin from cork. To present, beyond the publications enclosed in this thesis, only the study of Mattinen and co-workers[143] reported the use of 1-allyl-3-methylimidazolium chloride to solubilised suberin after its enzymatic depolymerisation from potato peels.

Herein, *Quercus suber* L. cork was selected as the main source of suberin not only due to the high amounts of readily available and misused industrial cork residues, but also due to its extreme importance in Portugal. The Mediterranean climate of the country offers exceptional conditions for the prosperous growth of the cork oak trees, which have a life span of around 250-350 years.[10] Cork processing, which includes

production, extraction and industrial transformation to value-added products, constitutes still today one of the most important economic sectors in Portugal. The Portuguese cork industry accounts for 50% of the world's total cork production, contributing for *ca.* 30% of the total exportation of Portuguese forestry products.[18]

For clarification proposes, a final word is essential regarding the selected suberin terminology used in the following chapters. There is "some controversy about the chemical delimitation of the term *suberin*."[10] Although it is known that suberised tissues comprise a polyaromatic and a polyaliphatic domain, some authors have proposed that the term suberin should be restricted to the polyaliphatic domain including its associated phenolics.[57] In fact, the depolymerisation procedures (see section 2.1) conventionally used to extract suberin from its natural sources only lead to the isolation of its polyaliphatic domain. In like manner, the ionic liquid herein used also lead to the isolation of the same suberin fraction, though with a different degree of polymerisation. Hence, in the present contribution the term suberin is applied towards the isolated fraction upon the ionic liquid depolymerisation, *i.e.* the aliphatic monomers and the associated phenolics.

As a cross-linked biopolyester, suberin cannot be dissolved without depolymerising, at least partially, its structure. For the sake of simplicity, along this thesis the term "suberin dissolution" was used referring to both processes: depolymerisation and concomitant dissolution of the ensuing structures. Accordingly, along this thesis the suberin extracted by cholinium hexanoate is referred to be partially depolymerised and cross-linked. In this scope, the term "cross-linking" was used to emphasise the partial preservation of suberin structure which most likely retain, at least partially, its native cross-linked and esterified structure exhibiting molecular weights that are high enough to render them insoluble in organic solvents. It is however recognized that, by definition, the dissolution of highly cross-linked macromolecular structures is not possible.

The subsequent chapters include the experimental approaches and results regarding the development of suberin films driven by an ionic liquid-based depolymerisation method. Ultimately, in the last chapter, an integrated discussion of all

the data herein described, as well as a critical analysis of the present contribution is provided.

5. References

1. R. C. Thompson, S. H. Swan, C. J. Moore and F. S. vom Saal, Our plastic age, *Philos. T. R. Soc. B*, 2009, **364**, 1973-1976.
2. M. N. Belgacem and A. Gandini, Monomers, Polymers and Composites from Renewable Resources, *Elsevier*, Amsterdam, The Netherlands, 1st edn., 2008.
3. APME, An analysis of plastics production, demand and recovery in Europe., *Brussels: Association of Plastics Manufacturers*, 2006.
4. S. Shafiee and E. Topal, When will fossil fuel reserves be diminished?, *Energ. Policy*, 2009, **37**, 181-189.
5. A. Gandini, Polymers from renewable resources: A challenge for the future of macromolecular materials, *Macromolecules*, 2008, **41**, 9491-9504.
6. A. J. Ragauskas, C. K. Williams, B. H. Davison, G. Britovsek, J. Cairney, C. A. Eckert, W. J. Frederick Jr., J. P. Hallett, D. J. Leak, C. L. Liotta, J. R. Mielenz, R. Murphy, R. Templer and T. Tschaplinski, The path forward for biofuels and biomaterials, *Science*, 2006, **311**, 484-489.
7. R. A. Gross and B. Kalra, Biodegradable polymers for the environment, *Science*, 2002, **297**, 803-807.
8. M. E. Himmel, S.-Y. Ding, D. K. Johnson, W. S. Adney, M. R. Nimlos, J. W. Brady and T. D. Foust, Biomass recalcitrance: Engineering plants and enzymes for biofuels production, *Science*, 2007, **315**, 804-807.
9. B. Kamm and M. Kamm, Principles of biorefineries, *Appl. Microbiol. Biot.*, 2004, **64**, 137-145.
10. H. Pereira, Cork: biology, production and uses, *Elsevier*, Amsterdam, The Netherlands, 1st edn., 2007.
11. K. Ranathunge, L. Schreiber and R. Franke, Suberin research in the genomics era—New interest for an old polymer, *Plant Sci.*, 2011, **180**, 399-413.
12. P. E. Kolattukudy, Polyesters in higher plants, in: *Biopolyesters*, ed. T. Scheper, W. Babel and A. Steinbuchel, 2001, vol. **71**, pp. 1-49.
13. O. Serra, S. Chatterjee, W. Huang and R. E. Stark, Mini-review: What nuclear magnetic resonance can tell us about protective tissues, *Plant Sci.*, 2012, **195**, 120-124.
14. L. Schreiber, Transport barriers made of cutin, suberin and associated waxes, *Trends Plant Sci.*, 2010, **15**, 546-553.
15. M. A. Bernards, Demystifying suberin, *Can. J. Bot.*, 2002, **80**, 227-240.
16. A. Gandini, C. Pascoal Neto and A. J. D. Silvestre, Suberin: A promising renewable resource for novel macromolecular materials, *Prog. Polym. Sci.*, 2006, **31**, 878-892.
17. P. C. R. O. Pinto, A. F. Sousa, A. J. D. Silvestre, C. Pascoal Neto, A. Gandini, C. Eckerman and B. Holmbom, *Quercus suber* and *Betula pendula* outer barks as renewable sources of oleochemicals: A comparative study, *Ind. Crop. Prod.*, 2009, **29**, 126-132.
18. APCOR, Cork, *http://www.apcor.pt/userfiles/File/Publicacoes/AnuarioAPCOR2012.pdf*, 2012.
19. P. A. Krasutsky, Birch bark research and development, *Nat. Prod. Rep.*, 2006, **23**, 919-942.
20. R. Ekman, The suberin monomers and triterpenoids from the outer bark of *Betula verrucosa* Ehrh., *Holzforschung*, 1983, **37**, 205-211.

21. L. Gil, Cortiça, produção tecnologia e aplicação, *INETI, Lisbon*, 1998.
22. A. Schieber, F. C. Stintzing and R. Carle, By-products of plant food processing as a source of functional compounds — recent developments, *Trends Food Sci. Tech.*, 2001, **12**, 401-413.
23. C. Israilides, A. G. Vlyssides, D. Arapoglou, T. Varzakas, R. Marchant and A. A. Vlysides, Integrated management of potato starch wastes, *Pr. Waste Resour. Manage.*, 2008.
24. A. F. Sousa, A. Gandini, A. J. D. Silvestre and C. Pascoal Neto, Synthesis and characterization of novel biopolyesters from suberin and model comonomers, *Chemsuschem*, 2008, **1**, 1020-1025.
25. A. F. Sousa, A. Gandini, A. J. D. Silvestre, C. Pascoal Neto, J. J. Cruz-Pinto, C. Eckerman and B. Holmbom, Novel suberin-based biopolyesters: From synthesis to properties, *J. Polym. Sci. Pol. Chem.*, 2011, **49**, 2281-2291.
26. A. F. Sousa, A. J. D. Silvestre, A. Gandini and C. Pascoal Neto, Synthesis of aliphatic suberin-like polyesters by ecofriendly catalytic systems, *High Perform. Polym.*, 2012, **24**, 4-8.
27. A. Schieber and M. D. A. Saldaña, Potato peels: A source of nutritionally and pharmacologically interesting compounds — a review, *Food*, 2009, **3**, 23-29.
28. M. H. Lopes, A. M. Gil, A. J. D. Silvestre and C. Pascoal Neto, Composition of suberin extracted upon gradual alkaline methanolysis of *Quercus suber* L. cork, *J. Agr. Food Chem.*, 2000, **48**, 383-391.
29. S. M. Rocha, B. J. Goodfellow, I. Delgadillo, C. Pascoal Neto and A. M. Gil, Enzymatic isolation and structural characterisation of polymeric suberin of cork from *Quercus suber* L., *Int. J. Biol. Macromol.*, 2001, **28**, 107-119.
30. P. J. Holloway, The composition of suberin from corks of *Quercus suber* L. and *Betula pendula* Roth, *Chem. Phys. Lipids*, 1972, **9**, 158-170.
31. P. E. Kolattukudy, Biopolyester membranes of plants: Cutin and suberin, *Science*, 1980, **208**, 990-1000.
32. P. E. Kolattukudy and B. B. Dean, Structure, gas chromatographic measurement, and function of suberin synthesized by potato tuber tissue slices, *Plant Physiol.*, 1974, **54**, 116-121.
33. P. J. Holloway and A. H. B. Deas, Epoxyoctadecanoic acids in plant cutins and suberins, *Phytochemistry*, 1973, **12**, 1721-1735.
34. J. Graça and S. Santos, Suberin: A biopolyester of plants' skin, *Macromol. Biosci.*, 2007, **7**, 128-135.
35. C. Coquet, E. Ferré, D. Peyronel, C. Dal Farra and A. M. Farnet, Identification of new molecules extracted from *Quercus suber* L. cork, *Cr. Biol.*, 2008, **331**, 853-858.
36. R. Ekman and C. Eckerman, Aliphatic carboxylic acids from suberin in birch outer bark by hydrolysis, methanolysis, and alkali fusion, *Pap. Puu-Pap. Tim.*, 1985, **67**, 255-273.
37. J. Negrel, F. Javelle and M. Paynot, Wound-induced tyramine hydroxycinnamoyl transferase in potato (*Solanum tuberosum*) tuber disks, *J. Plant Physiol.*, 1993, **142**, 518-524.
38. J. Graça and H. Pereira, Methanolysis of bark suberins: Analysis of glycerol and acid monomers, *Phytochem. Analysis*, 2000, **11**, 45-51.
39. S. Santos and J. Graça, Glycerol-ω-hydroxyacid-ferulic acid oligomers in cork suberin structure, *Holzforschung*, 2006, **60**, 171-177.
40. J. Graça and H. Pereira, Diglycerol alkenedioates in suberin: Building units of a poly(acylglycerol) polyester, *Biomacromolecules*, 2000, **1**, 519-522.
41. J. Graça and S. Santos, Glycerol-derived ester oligomers from cork suberin, *Chem. Phys. Lipids*, 2006, **144**, 96-107.
42. J. Graça and S. Santos, Linear aliphatic dimeric esters from cork suberin, *Biomacromolecules*, 2006, **7**, 2003-2010.
43. J. Graça and H. Pereira, Cork suberin: A glyceryl based polyester, *Holzforschung*, 1997, **51**, 225-234.

44. J. Graça and H. Pereira, Glyceryl-acyl and aryl-acyl dimers in *Pseudotsuga menziesii* bark suberin, *Holzforschung*, 1999, **53**, 397-402.
45. P. E. Kolattukudy and V. P. Agrawal, Structure and composition of aliphatic constituents of potato tuber skin (suberin), *Lipids*, 1974, **9**, 682-691.
46. M. F. Bento, H. Pereira, M. Á. Cunha, A. M. C. Moutinho, K. J. van den Berg and J. J. Boon, Thermally assisted transmethylation gas chromatography-mass spectrometry of suberin components in cork from *Quercus suber* L., *Phytochem. Analysis*, 1998, **9**, 75-87.
47. E. C. Lulai and W. C. Morgan, Histochemical probing of potato periderm with neutral red: A sensitive cytofluorochrome for the hydrophobic domain of suberin, *Biotech. Histochem.*, 1992, **67**, 185-195.
48. E. C. Lulai and D. L. Corsini, Differential deposition of suberin phenolic and aliphatic domains and their roles in resistance to infection during potato tuber (*Solanum tuberosum* L.) wound-healing, *Physiol. Mol. Plant P.*, 1998, **53**, 209-222.
49. J. Negrel, B. Pollet and C. Lapierre, Ether-linked ferulic acid amides in natural and wound periderms of potato tuber, *Phytochemistry*, 1996, **43**, 1195-1199.
50. M. H. Lopes, A. S. Barros, C. Pascoal Neto, D. Rutledge, I. Delgadillo and A. M. Gil, Variability of cork from portuguese *Quercus suber* studied by solid-state ^{13}C NMR and FTIR spectroscopies, *Biopolymers*, 2001, **62**, 268-277.
51. M. H. Lopes, C. Pascoal Neto, A. S. Barros, D. Rutledge, I. Delgadillo and A. M. Gil, Quantitation of aliphatic suberin in *Quercus suber* L. cork by FTIR spectroscopy and solid-state ^{13}C NMR spectroscopy, *Biopolymers*, 2000, **57**, 344-351.
52. R. E. Stark and J. R. Garbow, Nuclear magnetic resonance relaxation studies of plant polyester dynamics: 2. Suberized potato cell wall, *Macromolecules*, 1992, **25**, 149-154.
53. B. Yan and R. E. Stark, Biosynthesis, molecular structure, and domain architecture of potato suberin: A ^{13}C NMR study using isotopically labeled precursors, *J. Agr. Food Chem.*, 2000, **48**, 3298-3304.
54. M. A. Bernards, M. L. Lopez, J. Zajicek and N. G. Lewis, Hydroxycinnamic acid-derived polymers constitute the polyaromatic domain of suberin, *J. Biol. Chem.*, 1995, **270**, 7382-7386.
55. J. Graça, Hydroxycinnamates in suberin formation, *Phytochem. Rev.*, 2010, **9**, 85-91.
56. J. Graça and H. Pereira, Feruloyl esters of ω-hydroxyacids in cork suberin, *J. Wood Chem. Technol.*, 1998, **18**, 207-217.
57. J. Graça and H. Pereira, Suberin structure in potato periderm: Glycerol, long-chain monomers, and glyceryl and feruloyl dimers, *J. Agr. Food Chem.*, 2000, **48**, 5476-5483.
58. N. Cordeiro, M. N. Belgacem, A. J. D. Silvestre, C. Pascoal Neto and A. Gandini, Cork suberin as a new source of chemicals: 1. Isolation and chemical characterization of its composition, *Int. J. Biol. Macromol.*, 1998, **22**, 71-80.
59. E. W. Tegelaar, G. Hollman, P. Van der Vegt, J. W. De Leeuw and P. J. Holloway, Chemical characterization of the periderm tissue of some angiosperm species - recognition of an insoluble, nonhydrolyzable, aliphatic biomacromolecule (suberan), *Org. Geochem.*, 1995, **23**, 239-251.
60. J. W. Turner, B. E. Hartman and P. G. Hatcher, Structural characterization of suberan isolated from river birch (*Betula nigra*) bark, *Org. Geochem.*, 2013, **57**, 41-53.
61. R. G. Riley and P. E. Kolattukudy, Evidence for covalently attached *p*-coumaric acid and ferulic acid in cutins and suberins, *Plant Physiol.*, 1975, **56**, 650-654.
62. M. A. Bernards and F. A. Razem, The poly(phenolic) domain of potato suberin: A non-lignin cell wall bio-polymer, *Phytochemistry*, 2001, **57**, 1115-1122.

63. M. A. Bernards and N. G. Lewis, The macromolecular aromatic domain in suberized tissue: A changing paradigm, *Phytochemistry*, 1998, **47**, 915-933.
64. M. H. Lopes, A. Sarychev, C. Pascoal Neto and A. M. Gil, Spectral editing of ^{13}C CP/MAS NMR spectra of complex systems: Application to the structural characterisation of cork cell walls, *Solid State Nucl. Mag.*, 2000, **16**, 109-121.
65. P. Sitte, Zum Feinbau der Suberinschichten im Flaschenkork, *Protoplasma*, 1962, **54**, 555-559.
66. R. T. Teixeira and H. Pereira, Ultrastructural observations reveal the presence of channels between cork cells, *Microsc. Microanal.*, 2009, **15**, 539-544.
67. F. Beisson, Y. Li-Beisson and M. Pollard, Solving the puzzles of cutin and suberin polymer biosynthesis, *Curr. Opin. Plant Biol.*, 2012, **15**, 329-337.
68. N. Cordeiro, M. N. Belgacem, A. Gandini and C. Pascoal Neto, Urethanes and polyurethanes from suberin: 1. Kinetic study, *Ind. Crop. Prod.*, 1997, **6**, 163-167.
69. N. Cordeiro, M. N. Belgacem, A. Gandini and C. Pascoal Neto, Urethanes and polyurethanes from suberin 2: synthesis and characterization, *Ind. Crop. Prod.*, 1999, **10**, 1-10.
70. A. Olsson, M. Lindström and T. Iversen, Lipase-catalyzed synthesis of an epoxy-functionalized polyester from the suberin monomer *cis*-9,10-epoxy-18-hydroxyoctadecanoic acid, *Biomacromolecules*, 2007, **8**, 757-760.
71. N. Cordeiro, A. Blayo, N. M. Belgacem, A. Gandini, C. Pascoal Neto and J.-F. LeNest, Cork suberin as an additive in offset lithographic printing inks, *Ind. Crop. Prod.*, 2000, **11**, 63-71.
72. M. Evtiouguina, A. Barros-Timmons, J. J. Cruz-Pinto, C. P. Neto, M. N. Belgacem and A. Gandini, Oxypropylation of cork and the use of the ensuing polyols in polyurethane formulations, *Biomacromolecules*, 2002, **3**, 57-62.
73. M. Evtiouguina, A. M. Barros, J. J. Cruz-Pinto, C. Pascoal Neto, N. Belgacem, C. Pavier and A. Gandini, The oxypropylation of cork residues: Preliminary results, *Bioresource Technol.*, 2000, **73**, 187-189.
74. M. Evtiouguina, A. Gandini, C. Pascoal Neto and N. M. Belgacem, Urethanes and polyurethanes based on oxypropylated cork: 1. Appraisal and reactivity of products, *Polym. Int.*, 2001, **50**, 1150-1155.
75. L. Križková, M. H. Lopes, J. Polónyi, A. Belicová, J. Dobias and L. Ebringer, Antimutagenicity of a suberin extract from *Quercus suber* cork, *Mutat. Res.-Gen. Tox. En.*, 1999, **446**, 225-230.
76. P. J. Harris and L. R. Ferguson, Dietary fibres may protect or enhance carcinogenesis, *Mutat. Res.-Gen. Tox. En.*, 1999, **443**, 95-110.
77. C. Coquet, E. Bauza, G. Oberto, A. Berghi, A. M. Farnet, E. Ferré, D. Peyronel, C. Dal Farra and N. Domloge, *Quercus suber* cork extract displays a tensor and smoothing effect on human skin: An in vivo study, *Drug. Exp. Clin. Res.*, 2005, **31**, 89-99.
78. R. Franke and L. Schreiber, Suberin - a biopolyester forming apoplastic plant interfaces, *Curr. Opin. Plant Biol.*, 2007, **10**, 252-259.
79. Y. Li and F. Beisson, The biosynthesis of cutin and suberin as an alternative source of enzymes for the production of bio-based chemicals and materials, *Biochimie*, 2009, **91**, 685-691.
80. J. Graça, L. Schreiber, J. Rodrigues and H. Pereira, Glycerol and glyceryl esters of ω-hydroxyacids in cutins, *Phytochemistry*, 2002, **61**, 205-215.
81. J. Graça and P. Lamosa, Linear and branched poly(ω-hydroxyacid) esters in plant cutins, *J. Agr. Food Chem.*, 2010, **58**, 9666-9674.
82. J.-P. Douliez, J. Barrault, F. Jerome, A. Heredia, L. Navailles and F. Nallet, Glycerol derivatives of cutin and suberin monomers: Synthesis and self-assembly, *Biomacromolecules*, 2005, **6**, 30-34.

83. J. A. Heredia-Guerrero, A. Heredia, R. García-Segura and J. J. Benítez, Synthesis and characterization of a plant cutin mimetic polymer, *Polymer*, 2009, **50**, 5633-5637.
84. J. A. Heredia-Guerrero, M. A. San-Miguel, M. S. P. Sansom, A. Heredia and J. J. Benítez, Chemical Reactions in 2D: Self-assembly and self-esterification of 9(10),16-dihydroxypalmitic acid on mica surface, *Langmuir*, 2009, **25**, 6869-6874.
85. J. A. Heredia-Guerrero, M. A. San-Miguel, M. S. P. Sansom, A. Heredia and J. J. Benítez, Aleuritic (9,10,16-trihydroxypalmitic) acid self-assembly on mica, *Phys. Chem. Chem. Phys.*, 2010, **12**, 10423-10428.
86. S. Ghoshal, M. A. Khan, R. A. Khan, F. Gul-E-Noor and A. M. S. Chowdhury, Study on the thermo-mechanical and biodegradable properties of shellac films grafted with acrylic monomers by gamma radiation, *J. Polym. Environ.*, 2010, **18**, 216-223.
87. S. K. Nagappayya and V. G. Gaikar, Extraction of aleuritic acid from seedlac and purification by reactive adsorption on functionalized polymers, *Ind. Eng. Chem. Res.*, 2010, **49**, 6547-6553.
88. M. A. R. Meier, J. O. Metzger and U. S. Schubert, Plant oil renewable resources as green alternatives in polymer science, *Chem. Soc. Rev.*, 2007, **36**, 1788-1802.
89. J. W. Lee, H. U. Kim, S. Choi, J. Yi and S. Y. Lee, Microbial production of building block chemicals and polymers, *Curr. Opin. Biotech.*, 2011, **22**, 758-767.
90. P. Walden, Ueber die Molekulargrösse und elektrische Leitfähigkeit einiger geschmolzenen Salze, *Bull. Acad. Impér. Sci. St. Pétersbourg*, 1914, **8**, 405-422.
91. J. S. Wilkes and M. J. Zaworotko, Air and water stable 1-ethyl-3-methylimidazolium based ionic liquids, *J. Chem. Soc., Chem. Commun.*, 1992, 965-967.
92. N. V. Plechkova and K. R. Seddon, Applications of ionic liquids in the chemical industry, *Chem. Soc. Rev.*, 2008, **37**, 123-150.
93. W. L. Hough, M. Smiglak, H. Rodríguez, R. P. Swatloski, S. K. Spear, D. T. Daly, J. Pernak, J. E. Grisel, R. D. Carliss, M. D. Soutullo, J. H. J. Davis and R. D. Rogers, The third evolution of ionic liquids: Active pharmaceutical ingredients, *New J. Chem.*, 2007, **31**, 1429-1436.
94. J. N. A. Canongia Lopes and A. A. H. Pádua, Nanostructural organization in ionic liquids, *J. Phys. Chem. B*, 2006, **110**, 3330-3335.
95. J. N. Canongia Lopes, M. F. Costa Gomes and A. A. H. Pádua, Nonpolar, polar, and associating solutes in ionic liquids, *J. Phys. Chem. B*, 2006, **110**, 16816-16818.
96. L. P. N. Rebelo, J. N. C. Canongia Lopes, J. M. S. S. Esperança, H. J. R. Guedes, J. Łachwa, V. Najdanovic-Visak and Z. P. Visak, Accounting for the unique, doubly dual nature of ionic liquids from a molecular thermodynamic, and modeling standpoint, *Accounts Chem. Res.*, 2007, **40**, 1114-1121.
97. M. J. Earle, J. M. S. S. Esperança, M. A. Gilea, J. N. Canongia Lopes, L. P. N. Rebelo, J. W. Magee, K. R. Seddon and J. A. Widegren, The distillation and volatility of ionic liquids, *Nature*, 2006, **439**, 831-834.
98. M. Petkovic, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Ionic liquids: A pathway to environmental acceptability, *Chem. Soc. Rev.*, 2011, **40**, 1383-1403.
99. D. Coleman and N. Gathergood, Biodegradation studies of ionic liquids, *Chem. Soc. Rev.*, 2010, **39**, 600-637.
100. P. Anastas and N. Eghbali, Green chemistry: Principles and practice, *Chem. Soc. Rev.*, 2010, **39**, 301-312.
101. G. Imperato, B. König and C. Chiappe, Ionic green solvents from renewable resources, *Eur. J. Org. Chem.*, 2007, 1049-1058.

102. K. Fukumoto, M. Yoshizawa and H. Ohno, Room temperature ionic liquids from 20 natural amino acids, *J. Am. Chem. Soc.*, 2005, **127**, 2398-2399.
103. M. Petkovic, J. L. Ferguson, H. Q. N. Gunaratne, R. Ferreira, M. C. Leitão, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Novel biocompatible cholinium-based ionic liquids—toxicity and biodegradability, *Green Chem.*, 2010, **12**, 643-649.
104. R. Klein, O. Zech, E. Maurer, M. Kellermeier and W. Kunz, Oligoether carboxylates: Task-specific room-temperature ionic liquids, *J. Phys. Chem. B*, 2011, **115**, 8961-8969.
105. Y. Fukaya, Y. Iizuka, K. Sekikawa and H. Ohno, Bio ionic liquids: Room temperature ionic liquids composed wholly of biomaterials, *Green Chem.*, 2007, **9**, 1155-1157.
106. E. B. Carter, S. L. Culver, P. A. Fox, R. D. Goode, I. Ntai, M. D. Tickell, R. K. Traylor, N. W. Hoffman and J. H. J. Davis, Sweet success: Ionic liquids derived from non-nutritive sweeteners, *Chem. Commun.*, 2004, 630-631.
107. L. Poletti, C. Chiappe, L. Lay, D. Pieraccini, L. Polito and G. Russo, Glucose-derived ionic liquids: Exploring low-cost sources for novel chiral solvents, *Green Chem.*, 2007, **9**, 337-341.
108. A. P. Abbott, G. Capper, D. L. Davies, H. L. Munro, R. K. Rasheed and V. Tambyrajah, Preparation of novel, moisture-stable, Lewis-acidic ionic liquids containing quaternary ammonium salts with functional side chains, *Chem. Commun.*, 2001, 2010-2011.
109. J. K. Blusztajn, Choline, a vital amine, *Science*, 1998, **281**, 794-795.
110. K. Haerens, E. Matthijs, A. Chmielarz and B. Van der Bruggen, The use of ionic liquids based on choline chloride for metal deposition: A green alternative?, *J. Environ. Manage.*, 2009, **90**, 3245-3252.
111. A. P. Abbott, K. S. Ryder and U. Knig, Electrofinishing of metals using eutectic based ionic liquids, *T. I. Met. Finish.*, 2008, **86**, 196-204.
112. B. Jastorff, R. Störmann, J. Ranke, K. Mölter, F. Stock, B. Oberheitmann, W. Hoffmann, J. Hoffmann, M. Nüchter, B. Ondruschka and J. Filser, How hazardous are ionic liquids? Structure-activity relationships and biological testing as important elements for sustainability evaluation, *Green Chem.*, 2003, **5**, 136-142.
113. M. Armand, F. Endres, D. R. MacFarlane, H. Ohno and B. Scrosati, Ionic-liquid materials for the electrochemical challenges of the future, *Nat. Mater.*, 2009, **8**, 621-629.
114. R. A. Sheldon, Green solvents for sustainable organic synthesis: State of the art, *Green Chem.*, 2005, **7**, 267-278.
115. J. P. Hallett and T. Welton, Room-temperature ionic liquids: Solvents for synthesis and catalysis. 2, *Chem. Rev.*, 2011, **111**, 3508-3576.
116. X. Han and D. W. Armstrong, Ionic liquids in separations, *Accounts Chem. Res.*, 2007, **40**, 1079-1086.
117. A. Brandt, J. Gräsvik, J. P. Hallett and T. Welton, Deconstruction of lignocellulosic biomass with ionic liquids, *Green Chem.*, 2013, **15**, 550-583.
118. N. Sun, H. Rodríguez, M. Rahman and R. D. Rogers, Where are ionic liquid strategies most suited in the pursuit of chemicals and energy from lignocellulosic biomass?, *Chem. Commun.*, 2011, **47**, 1405-1421.
119. K. Fujita, D. R. MacFarlane and M. Forsyth, Protein solubilising and stabilising ionic liquids, *Chem. Commun.*, 2005, 4804-4806.
120. H. Wang, G. Gurau and R. D. Rogers, Ionic liquid processing of cellulose, *Chem. Soc. Rev.*, 2012, **41**, 1519-1537.
121. M. M. Hossain and L. Aldous, Ionic liquids for lignin processing: Dissolution, isolation, and conversion, *Aust. J. Chem.*, 2012, **65**, 1465-1477.

122. A. Pinkert, K. N. Marsh, S. Pang and M. P. Staiger, Ionic liquids and their interaction with cellulose, *Chem. Rev.*, 2009, **109**, 6712-6728.

123. N. Sun, M. Rahman, Y. Qin, M. L. Maxim, H. Rodríguez and R. D. Rogers, Complete dissolution and partial delignification of wood in the ionic liquid 1-ethyl-3-methylimidazolium acetate, *Green Chem.*, 2009, **11**, 646-655.

124. D. Fu and G. Mazza, Aqueous ionic liquid pretreatment of straw, *Bioresource Technol.*, 2011, **102**, 7008-7011.

125. I. Kilpeläinen, H. Xie, A. King, M. Granstrom, S. Heikkinen and D. S. Argyropoulos, Dissolution of wood in ionic liquids, *J. Agr. Food Chem.*, 2007, **55**, 9142-9148.

126. D. A. Fort, R. C. Remsing, R. P. Swatloski, P. Moyna, G. Moyna and R. D. Rogers, Can ionic liquids dissolve wood? Processing and analysis of lignocellulosic materials with 1-*n*-butyl-3-methylimidazolium chloride, *Green Chem.*, 2007, **9**, 63-69.

127. G. Cheng, P. Varanasi, C. Li, H. B. Liu, Y. B. Menichenko, B. A. Simmons, M. S. Kent and S. Singh, Transition of cellulose crystalline structure and surface morphology of biomass as a function of ionic liquid pretreatment and its relation to enzymatic hydrolysis, *Biomacromolecules*, 2011, **12**, 933-941.

128. S. Singh, B. A. Simmons and K. P. Vogel, Visualization of biomass solubilization and cellulose regeneration during ionic liquid pretreatment of switchgrass, *Biotechnol. Bioeng.*, 2009, **104**, 68-75.

129. R. P. Swatloski, S. K. Spear, J. D. Holbrey and R. D. Rogers, Dissolution of cellose with ionic liquids, *J. Am. Chem. Soc.*, 2002, **124**, 4974-4975.

130. S. H. Lee, T. V. Doherty, R. J. Linhardt and J. S. Dordick, Ionic liquid-mediated selective extraction of lignin from wood leading to enhanced enzymatic cellulose hydrolysis, *Biotechnol. Bioeng.*, 2009, **102**, 1368-1376.

131. R. C. Remsing, G. Hernandez, R. P. Swatloski, W. W. Massefski, R. D. Rogers and G. Moyna, Solvation of carbohydrates in *N,N'*-dialkylimidazolium ionic liquids: A multinuclear NMR spectroscopy study, *J. Phys. Chem. B*, 2008, **112**, 11071-11078.

132. H. Liu, K. L. Sale, B. M. Holmes, B. A. Simmons and S. Singh, Understanding the interactions of cellulose with ionic liquids: A molecular dynamics study, *J. Phys. Chem. B*, 2010, **114**, 4293-4301.

133. A. S. Gross, A. T. Bell and J.-W. Chu, Thermodynamics of cellulose solvation in water and the ionic liquid 1-butyl-3-methylimidazolim chloride, *J. Phys. Chem. B*, 2011, **115**, 13433-13440.

134. M. Abe, Y. Fukaya and H. Ohno, Fast and facile dissolution of cellulose with tetrabutylphosphonium hydroxide containing 40 wt% water, *Chem. Commun.*, 2012, **48**, 1808-1810.

135. H. Zhao, J. E. Holladay, H. Brown and Z. C. Zhang, Metal chlorides in ionic liquid solvents convert sugars to 5-hydroxymethylfurfural, *Science*, 2007, **316**, 1597-1600.

136. H. Xie, S. Zhang and S. Li, Chitin and chitosan dissolved in ionic liquids as reversible sorbents of CO_2, *Green Chem.*, 2006, **8**, 630-633.

137. A. Idris, R. Vijayaraghavan, U. A. Rana, D. Fredericks, A. F. Patti and D. R. MacFarlane, Dissolution of feather keratin in ionic liquids, *Green Chem.*, 2013, **15**, 525-534.

138. H. Xie, S. Li and S. Zhang, Ionic liquids as novel solvents for the dissolution and blending of wool keratin fibers, *Green Chem.*, 2005, **7**, 606-608.

139. A. Biswas, R. L. Shogren, D. G. Stevenson, J. L. Willett and P. K. Bhowmik, Ionic liquids as solvents for biopolymers: Acylation of starch and zein protein, *Carbohyd. Polym.*, 2006, **66**, 546-550.

140. Q. Wu, H. Chen, M. Han, D. Wang and J. Wang, Transesterification of cottonseed oil catalyzed by bronsted acidic ionic liquids, *Ind. Eng. Chem. Res.*, 2007, **46**, 7955-7960.

141. A. H. M. Fauzi and N. A. S. Amin, An overview of ionic liquids as solvents in biodiesel synthesis, *Renew. Sust. Energ. Rev.*, 2012, **16**, 5770-5786.

142. R. E. Teixeira, Energy-efficient extraction of fuel and chemical feedstocks from algae, *Green Chem.*, 2012, **14**, 419-427.

143. M.-L. Mattinen, I. Filpponen, R. Järvinen, B. Li, H. Kallio, P. Lehtinen and D. Argyropoulos, Structure of the polyphenolic component of suberin isolated from potato (*Solanum tuberosum* var. Nikola), *J. Agr. Food Chem.*, 2009, **57**, 9747-9753.

Chapter II

Dissolution of cork biopolymers in biocompatible ionic liquids

Adapted from: H. Garcia, R. Ferreira, M. Petkovic, J. L. Ferguson, M. C. Leitão, H. Q. Nimal Gunaratne, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Dissolution of cork biopolymers by biocompatible ionic liquids, *Green Chem.*, 2010, **12**, 367 – 369.

The author had contributed to the planning and execution of all the depolymerisation reactions and spectroscopic characterisation herein presented, as well as on the discussion, interpretation and preparation of the manuscript.

1. Abstract

Classically, the best attempts to separate suberin from cork biopolymers have been attained by conventional hydrolysis and methanolysis processes; here, we report a class of biocompatible and biodegradable cholinium-based ionic liquids, the cholinium alkanoates, which show a highly efficient and specific dissolution of suberin from cork.

2. Communication

Worldwide, the annual production of cork, which is the external bark of *Quercus suber* L., is 300 000 tonnes, half of which forms the basis of the Portuguese cork manufacturing industries.[1] Cork is a remarkable biocomposite, showing a very specific combination of properties, such as elasticity, compressibility, low permeability for liquids, and significant chemical/microbial resistance;[2] there is thus a significant interest in suberin (its major component) as a valuable source of property-enhancing additives[3]. The conventional pre-treatment of cork[4] removes the soluble components (extractives), but leaves an essentially insoluble matrix (extractive-free, or refined, cork) whose main components are polysaccharides, lignin and suberin (~20, ~30 and ~50 wt %, respectively).[5] Suberin is a complex cross-linked polymer, composed of aromatic and aliphatic domains.[6] The former domain is relatively similar to lignin, and possesses a very complex structure comprising units of hydroxycinnamic acid and, to a minor extent, monolignols (*p*-coumaryl, coniferyl, and sinapyl alcohols); the latter domain is composed mostly of units of C_{16}-C_{26} hydroxyacids that are primarily linked, *via* ester bonds, to glycerol.[2,7] This means that, structurally, cork has a strong relationship with lignocellulosic materials.

Ionic liquids exhibit a set of unique and astonishing properties, such as negligible vapour pressure, bulk non-flammability, and high thermal and chemical stability. Because their thermophysical and chemical properties can be fine-tuned through slight alterations to the cations, anions or both, they can address very specific requirements.[8] A major industrial interest in ionic liquids is the dissolution and processing of cellulose, which has been successfully achieved with some 1,3-dialkylimidazolium ionic liquids.[9,10]

The basis of their outstanding solvation behaviour results from their Coulombic environment,[8] and is thought to be due to an ionic liquid's ability to disrupt intermolecular hydrogen-bonding networks and interact with the hydroxyl groups of cellulose.[9,11] However, the scope of possible applications of 1,3-dialkylimidazolium ionic liquids is restricted due to their cost, toxicity[12] and/or environmental persistence due to the non-biodegradability[13] of the imidazolium ring.

Refined cork has been reported to be insoluble in many common solvents,[3] but its apparent similarities with other lignocellulosic materials suggest that it might be soluble in tailor-made ionic liquids. Here, we report the first example of refined cork dissolution by ionic liquids. Moreover, the ionic liquids selected are both biocompatible and biodegradable.

Learning from both the previous experience of the dissolution of lignocellulosic materials, and the extant toxicological and biodegradation data of ionic liquid cations and anions, a test group of ionic liquids was selected by combining three different cations (1-ethyl-3-methylimidazolium, $[C_2mim]^+$, 1-butyl-3-methylimidazolium, $[C_4mim]^+$, and cholinium, $[N_{111}C_2H_4OH]^+$) and six different anions. The effect of the anion was studied focussing initially on chloride, ethanoate and lactate anions, and, in the case of the cholinium ionic liquids,[14,15] on a further three alkanoates.

Cork samples were initially ground to a fine powder, and the soluble cork extracts (extractives) were removed as previously described by Gil *et al.*[4] Prior to the dissolution tests (all performed in triplicate), the ionic liquid and the refined cork powder were vacuum dried in order to remove water. The ionic liquid (*ca.* 1.6 g) was added to the refined cork powder (*ca.* 0.025 g) (ionic liquid:cork ≈ 2:1 v/v) and the mixture held at 100 ºC (which is below the degradation temperature of all the ionic liquids) without any stirring for 4 h. The dissolution was then stopped by adding an excess of de-ionised water (*ca.* 20 cm^3), and the insoluble residue was recovered by filtration and dried. Under these conditions, in separate experiments, no significant decomposition was detected for either the cork or the ionic liquids.

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy has successfully been used for cork characterisation, and consequently it has been

selected here for qualitative assessment of the dissolution process.[2,5,6] These studies determined quite precisely the infrared absorption features of the primary components of refined cork:

Suberin: 2921, 2852, 1737, 1242, 1158 and 724 cm^{-1}

Lignin: 1511, 855 and 819 cm^{-1}

Polysaccharides: 1092 and 1034 cm^{-1}

The samples of refined cork powder prepared for this study, both before and after heating (control), correspond exactly to these literature data.

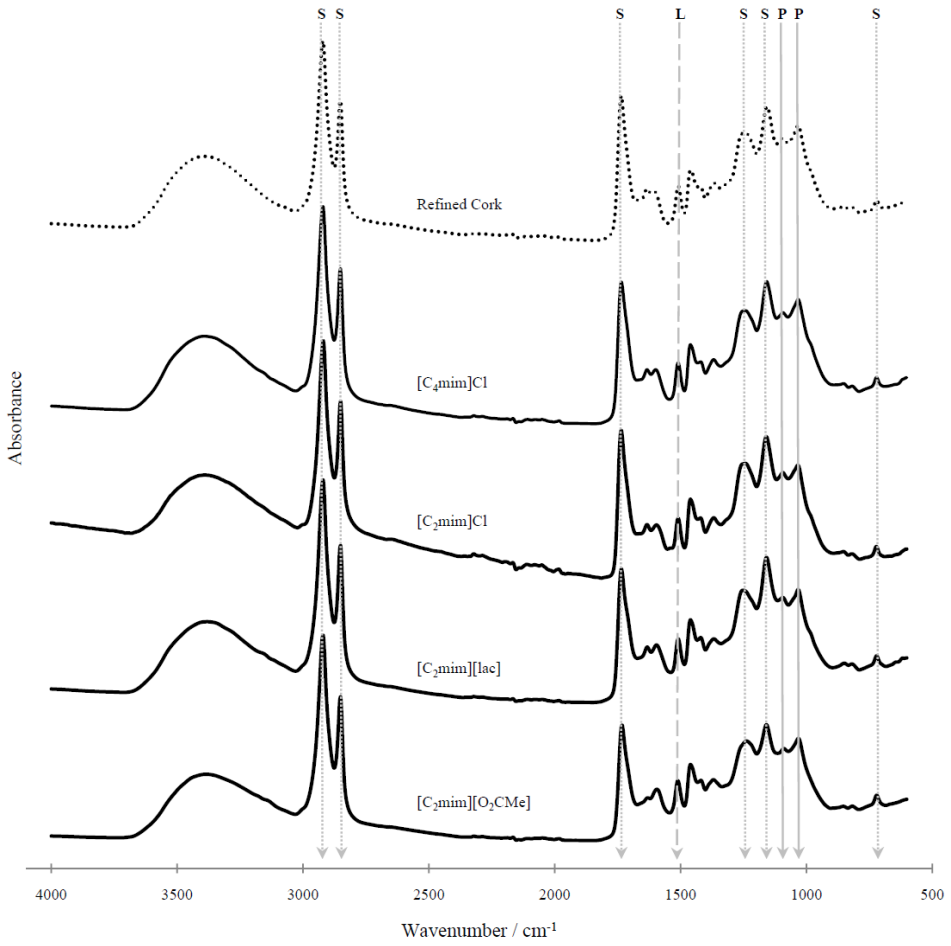


Figure 1| ATR-FTIR spectra of the insoluble cork fraction after treatment with different 1,3-dialkylimidazolium ionic liquids. The vertical lines identify the peaks mainly assigned to suberin (S), lignin (L) and polysaccharides (P).

ATR-FTIR analyses of the cork residue after its extraction by ionic liquids from the 1,3-dialkylimidazolium family are presented in Figure 1. Neither $[C_2mim]Cl$ nor $[C_4mim]Cl$ were able to dissolve significant amounts of the refined cork, even though they have been previously observed to enable significant dissolution of polysaccharides in lignocellulosic composites.[11,16] However, replacing the chloride with various carboxylate anions, namely lactate, $[lac]^-$, and ethanoate, caused the dissolution efficiency to increase significantly. The latter was more efficient and led to a greater dissolution of suberin (Figure 1, shown by the decreasing intensity of the peaks at 1737, 1242 and 1158 cm^{-1}). Both anions led to a small solubility of the polysaccharides, as illustrated by the changes in the intensity of the peaks at 1092 and 1034 cm^{-1}.

The results for the cholinium, $[Me_3NCH_2CH_2OH]^+$ or $[N_{111}C_2H_4OH]^+$, salts are presented in Figure 2 and Table 1. The cholinium ethanoate showed, relative to the 1,3-dialkylimidazolium ionic liquids, a significant increase in the dissolution of the refined cork. The lack of efficiency of the lactate anion, relative to the ethanoate (cork weight losses of 20.7 and 39.7 %, respectively), is unsurprising, as the lactate anion is less basic than the ethanoate anion. Further support for this simple concept is that the cholinium alkanoates (ethanoate, butanoate, hexanoate), showed augmented dissolution efficiency due to the increasing basicity and chain length of the anion (Table 1). The efficiency of the anions can be ranked as follows:

$$[lac]^- < [O_2CMe]^- < [O_2CPr]^- < [O_2CCHMe_2]^- < [O_2CC_5H_{11}]^-$$

Table 1| Data on the dissolution efficiency of refined cork in a range of cholinium alkanoates, $[N_{111}C_2H_4OH]Y$. The pK_a values of the conjugate acid of the anion of each ionic liquid is also presented.

Y^-	E_{Solv} (%)[a]	STD[b]	pK_a (HY)
$[O_2CMe]^-$	39.7	2.5	4.76
$[O_2CPr]^-$	44.1	10.8	4.84
$[O_2CCHMe_2]^-$	55.1	5.8	4.83
$[O_2CC_5H_{11}]^-$	64.9	7.9	4.85
$[lac]^-$	20.7	4.1	3.86

[a] Dissolution efficiency, E_{solv} (%) = 100 × ($m_{cork,\ t=0} - m_{cork,\ t=4h}$) / $m_{cork,\ t=0}$
[b] STD = standard deviation

The dissolution efficiency tracks the pK_a values of the conjugate acid of the anion, chloride being the least efficient. This goes in line with the dissolution of cellulose in ionic liquids, where the disruption of the hydrogen-bond network is also associated with the basicity of the anion.[17]

The dissolution of cork by $[N_{111}C_2H_4OH][O_2CMe]$, $[N_{111}C_2H_4OH][O_2CPr]$, $[N_{111}C_2H_4OH][O_2CCHMe_2]$, and $[N_{111}C_2H_4OH][O_2CC_5H_{11}]$ resulted, progressively, in a remarkable reduction (Figure 2) of the intensity of the peaks assigned to suberin (2921, 2852, 1735, 1242, 1158 and 724 cm^{-1}) in the insoluble cork residue. The peak assigned at 1735 cm^{-1} (associated with the carbonyl stretch of the ester groups) was virtually absent in the case of hexanoate, with concomitant formation of a small shoulder at 1712 cm^{-1}. This effect is possibly associated with the formation of acidic groups resulting from suberin removal.[5]

To eliminate the possibility that dissolution was due to hydrolysis of the ionic liquid, control tests were performed using the conjugate acids of the ionic liquids' anions. Butanoic and hexanoic acids (pure and dried with molecular sieves) were tested, resulting in a weight loss of 10.3 % and 17.2 % respectively, and no evident alterations were observed in ATR-FTIR spectra.

The cholinium family of ionic liquids present a very low toxicity,[18] especially to some eukaryotic organisms (*e.g.* ref [19]), due to the benign nature of the cation and its high biodegradability potential.[20] The toxicity (*i.e.*, growth inhibition effect[19]) of $[N_{111}C_2H_4OH][O_2CPr]$, $[N_{111}C_2H_4OH][O_2CCHMe_2]$, and $[N_{111}C_2H_4OH][O_2CC_5H_{11}]$ towards *P. corylophilum*, which has been previously demonstrated to show a moderate susceptibility to several ionic liquids,[19] were evaluated here. They exhibited very low inhibitory capacities, with MIC values of 150, 250 and 62.5 mM, respectively. The biodegradation of the most efficient system was then evaluated under aerobic conditions. After four weeks of incubation, the residual concentration of $[O_2CC_5H_{11}]^-$ in *P. corylophilum* cultures was <12 %, as determined by liquid chromatography. The biodegradation of the anion was confirmed by ^{1}H NMR spectroscopy and shown by the disappearance of the anion peaks in the spectra relative to the cation.

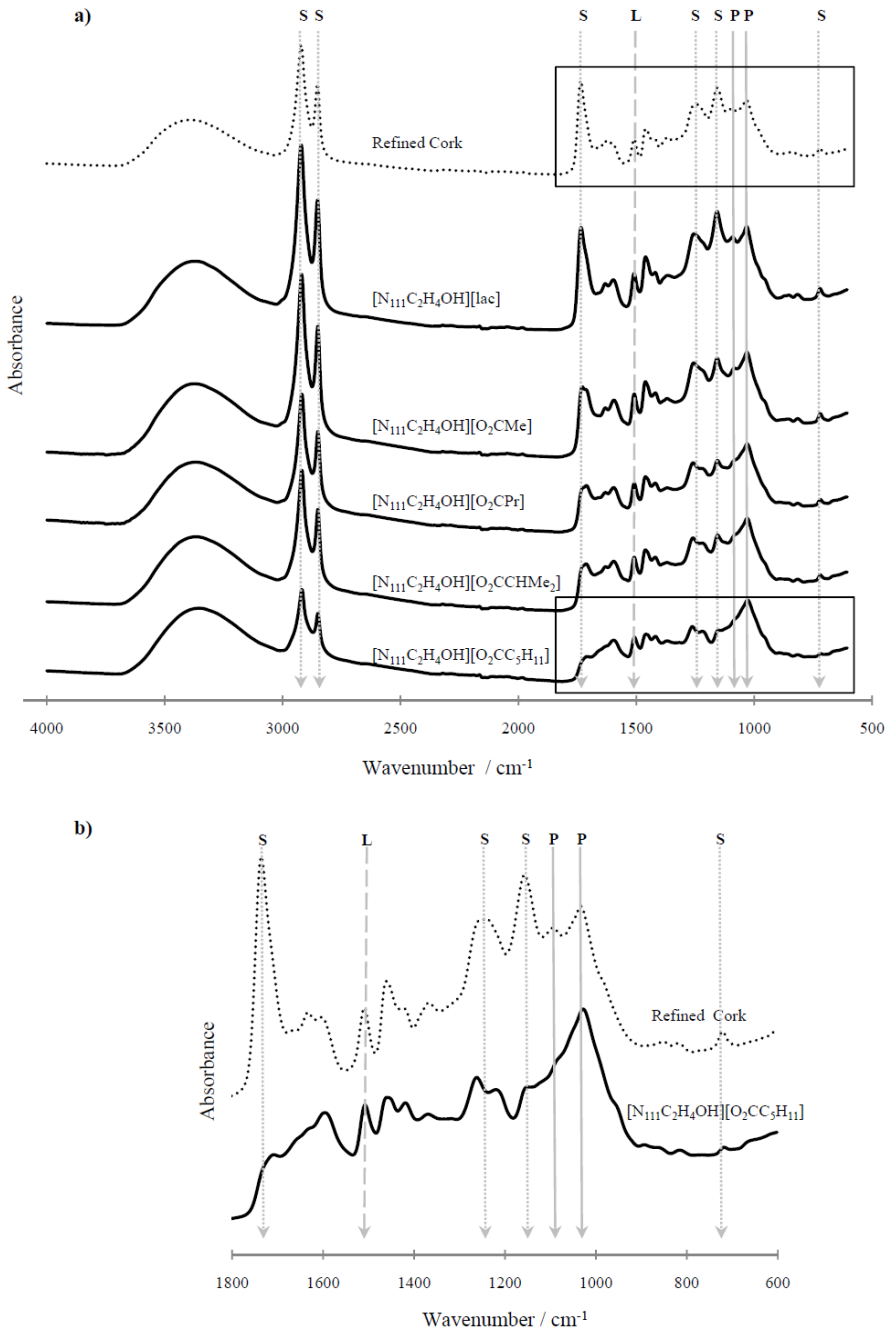


Figure 2| (**a**) ATR-FTIR spectra of the cork residue after treatment with different cholinium ionic liquids. (**b**) An expansion of the ATR-FTIR spectra of the cork residue after treatment with cholinium hexanoate. The vertical lines identify the peaks mainly assigned to suberin (S), lignin (L) and polysaccharides (P).

The biocompatible and biodegradable cholinium hexanoate appears to be the most promising ionic liquid for refined cork dissolution, particularly with respect to the separation of large quantities of suberin. Taking advantage of their synthetic tuneability,[8] there is clearly a huge potential for developing and exploring even more efficient systems.

3. Acknowledgments

R.F. is grateful to FC&T for the fellowship SFRH/BD/48286/2008, and H.G. is indebted to FCG for the fellowship 21-95587-B. The work was partially supported by a grant from Iceland, Liechtenstein and Norway through the EEA financial mechanism (Project PT015) and by FC&T (Project REDE/1504/REM/2005). The authors wish to acknowledge Dr. M. Vitória San Romão for providing the cork powder used in this study. J.L.F. and K.R.S. wish to thank the QUILL Industrial Advisory Board and the EPSRC (Portfolio Partnership Scheme, grant number EP/D029538/1) for their continued support.

4. References

1. J. A. Maga and J. L. Puech, Cork and alcoholic beverages, *Food Rev. Int.*, 2005, **21**, 53-68.
2. M. H. Lopes, A. S. Barros, C. Pascoal Neto, D. Rutledge, I. Delgadillo and A. M. Gil, Variability of cork from portuguese *Quercus suber* studied by solid-state ^{13}C NMR and FTIR spectroscopies, *Biopolymers*, 2001, **62**, 268-277.
3. N. Cordeiro, N. M. Belgacem, A. Gandini and C. Pascoal Neto, Cork suberin as a new source of chemicals: 2. Crystallinity, thermal and rheological properties, *Bioresource Technol.*, 1998, **63**, 153-158.
4. A. M. Gil, M. Lopes, J. Rocha and C. Pascoal Neto, A ^{13}C solid state nuclear magnetic resonance spectroscopic study of cork cell wall structure: The effect of suberin removal., *Int. J. Biol. Macromol.*, 1997, **20**, 293-305.
5. C. Pascoal Neto, J. Rocha, A. Gil, N. Cordeiro, A. P. Esculcas, S. Rocha, I. Delgadillo, J. D. Pedrosa de Jesus and A. J. Ferrer Correia, ^{13}C solid-state nuclear magnetic resonance and *Fourier* transform infrared studies of the thermal decomposition of cork, *Solid State Nucl. Mag.*, 1995, **4**, 143-151.
6. M. H. Lopes, C. Pascoal Neto, A. S. Barros, D. Rutledge, I. Delgadillo and A. M. Gil, Quantitation of aliphatic suberin in *Quercus suber* L. cork by FTIR spectroscopy and solid-state ^{13}C NMR spectroscopy, *Biopolymers*, 2000, **57**, 344-351.
7. M. A. Bernards, Demystifying suberin, *Can. J. Bot.*, 2002, **80(3)**, 227–240.
8. A. Stark and K. R. Seddon, Ionic Liquids, in: *Kirk-Othmer Encyclopaedia of Chemical Technology*, ed. A. Seidel, John Wiley & Sons, Inc., Hoboken, New Jersey, USA, 5th edn., 2007, vol. **26**, pp. 836-920.

9. D. A. Fort, R. C. Remsing, R. P. Swatloski, P. Moyna, G. Moyna and R. D. Rogers, Can ionic liquids dissolve wood? Processing and analysis of lignocellulosic materials with 1-*n*-butyl-3-methylimidazolium chloride, *Green Chem.*, 2007, **9**, 63-69.
10. Y. Fukaya, K. Hayashi, M. Wada and H. Ohno, Cellulose dissolution with polar ionic liquids under mild conditions: Required factors for anions, *Green Chem.*, 2008, **10**, 44-46.
11. I. Kilpeläinen, H. Xie, A. King, M. Granstrom, S. Heikkinen and D. S. Argyropoulos, Dissolution of wood in ionic liquids, *J. Agr. Food Chem.*, 2007, **55**, 9142-9148.
12. P. Stepnowski, A. C. Składanowski, A. Ludwiczak and E. Łaczyńska, Evaluating the cytotoxicity of ionic liquids using human cell line HeLa, *Hum. Exp. Toxicol.*, 2004, **23**, 513-517.
13. S. Stolte, S. Abdulkarim, J. Arning, A.-K. Blomeyer-Nienstedt, U. Bottin-Weber, M. Matzke, J. Ranke, B. Jastorff and J. Thöming, Primary biodegradation of ionic liquid cations, identification of degradation products of 1-methyl-3-octylimidazolium chloride and electrochemical wastewater treatment of poorly biodegradable compounds, *Green Chem.*, 2008, **10**, 214-224.
14. A. P. Abbott and D. L. Davies, Ionic liquids prepared as low melting salts and compounds of quaternary ammonium halides with metal halides, *World Pat.*, WO 0056700, 2000.
15. A. P. Abbott, D. L. Davies, G. Capper, R. K. Rasheed and V. Tambyrajah, Ionic liquids and their use as solvents, *World Pat.*, WO 2002 026701, 2002.
16. R. P. Swatloski, S. K. Spear, J. D. Holbrey and R. D. Rogers, Dissolution of cellulose with ionic liquids, *J. Am. Chem. Soc.*, 2002, **124**, 4974-4975.
17. N. Sun, M. Rahman, Y. Qin, M. L. Maxim, H. Rodríguez and R. D. Rogers, Complete dissolution and partial delignification of wood in the ionic liquid 1-ethyl-3-methylimidazolium acetate, *Green Chem.*, 2009, **11**, 646-655.
18. P. Nockemann, B. Thijs, K. Driesen, C. R. Janssen, K. Van Hecke, L. Van Meervelt, S. Kossmann, B. Kirchner and K. Binnemans, Choline saccharinate and choline acesulfamate: Ionic liquids with low toxicities, *J. Phys. Chem. B*, 2007, **111**, 5254-5263.
19. M. Petkovic, J. Ferguson, A. Bohn, J. R. Trindade, I. Martins, M. Carvalho, M. C. Leitão, C. Rodrigues, H. Garcia, R. Ferreira, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Exploring fungal activity in the presence of ionic liquids, *Green Chem.*, 2009, **11**, 889–894.
20. R. S. Boethling, E. Sommer and D. DiFiore, Designing small molecules for biodegradability, *Chem. Rev.*, 2007, **107**, 2207-2227.

Chapter III

Suberin isolation from cork using ionic liquids: characterisation of ensuing products

Adapted from: H. Garcia, R. Ferreira, A. F. Sousa, M. Petkovic, P. Lamosa, C. S. R. Freire, A. J. D. Silvestre, L. P. N. Rebelo, and C. Silva Pereira, Suberin isolation process from cork using ionic liquids: Characterisation of ensuing products, *New J. Chem.*, 2012, **36**, 2014–2024.

The author had contributed to the planning and execution of all the experiments herein presented, as well as on the discussion, interpretation and preparation of the manuscript. SEM, EA, solid state NMR, GC-MS and DMA analyses were performed in collaboration with technicians or co-authors.

1. Abstract

Cholinium alkanoates, a class of benign ionic liquids, were demonstrated to efficiently extract suberin from cork. A detailed characterisation of the extracted material has yet to be attained. In the present study the significance of the alkylic chain length of the anion and the ionic liquid's basicity was investigated. The results obtained emphasise cholinium hexanoate's selection; it proved to be a straightforward process, also ensuring the recyclability and reusability of the ionic liquid. The extracted suberinic material has been thoroughly characterised for the first time by ATR-FTIR, NMR, GC-MS and thermal analyses. Data showed that it is mainly composed of oligomeric or polymeric aliphatic esterified structures, resulting from suberin partial cleavage. More than 40 wt % of the extracted suberinic material was found to be cross-linked. Even though, the composing monomeric units were similar to those usually identified in suberin samples obtained by the conventional extraction processes. These data pave the way for advanced studies of suberin monomers/oligomers as building-blocks for the development of novel biopolymers and biomaterials.

Keywords**:** cholinium hexanoate, cork, ionic liquids, renewable resources, suberin.

2. Introduction

Biomass feedstocks constitute a source of numerous value-added compounds, such as biopolymers, biofuels, and building-block chemicals.[1] Cork, the outer bark of *Quercus suber* L., is a remarkable plant composite displaying a very specific combination of properties, such as elasticity, compressibility, low density, low permeability, and significant chemical and microbial resistance.[2] Historically, cork utility goes back to the ancient Romans, and since then has been used essentially to manufacture stoppers and thermal/sound insulation materials. Globally, ≥ 300 000 tonnes of cork are processed *per annum* by industry, generating large amounts of residues (*ca.* 22 wt %), especially cork of small grains size, which, despite its interesting chemical composition, is generally burned to produce energy.[3]

Cork is composed of suberin, lignin, polysaccharides, and extractives (approximately 50, 20, 20 and 10 wt %, respectively).[4-6] Suberin, an aromatic-aliphatic cross-linked biopolyester, represents *per se* a source of property-enhancing additives.[7] It is a three-dimensional complex network occurring in the secondary plant cell wall. While its composition and native structural organisation are still controversial, the domains of suberin are generally thought to be arranged in a lamellar-type structure.[5,7-10] The aliphatic domain is composed mostly of even numbered units (C_{16}-C_{26}) of ω-hydroxyalkanoic and α,ω-alkanedioic acids (some with *mid*-chain unsaturation, epoxy or *vic*-diol functionalities), alkanoic acids and aliphatic alcohols. The aromatic domain shows a quite distinctive composition, with some similarities to lignin, predominantly composed of hydroxycinnamic acid units, with residual amounts of *p*-coumaryl, coniferyl, and sinapyl alcohols.[5,6,8,11-13] The suberin monomers are cross-linked *via* ester bonds involving glycerol units or aliphatic hydroxyl and carboxylic moieties.[12,14] However, the nature of the linking of suberin to the other cell wall domains remains uncertain.[15]

Depolymerisation of *in-situ* suberin and its simultaneous isolation from the plant composite is traditionally a laborious process requiring harsh chemical processes.[7] These processes involve extensive ester bond cleavage, normally attained through alkaline methanolysis with sodium methoxide,[5,11,12,16,17] or alkaline hydrolysis.[18] Furthermore, suberin partial depolymerisation, leading to the formation of oligomeric structures, can be achieved using more gentle processes although with limited extraction yields, *e.g.* methanolysis catalysed by calcium oxide.[19]

The last few decades have witnessed an exponential growth of interest in ionic liquids – a disparate class of chemicals composed solely of ions that are liquid below a temperature conventionally defined as 100 °C.[20] Several hundred ionic liquids are already available and characterised,[21] and one can reasonably estimate that millions of cation/anion combinations are possible. Furthermore, fine-tuning of the cation and/or the anion might be used to address very specific thermophysical and chemical properties,[20] and even biological activity.[22] Ionic liquids usually exhibit a set of remarkable features, such as negligible vapour pressure,[23] bulk non-flammability, thermal stability, and high solvent ability.[20] The latter characteristic arises from the combination of the organic

functionalities of the ions with the Coulombic environment created by them, resulting in a structural arrangement of charged and apolar micro-domains.[24,25] A combination of the aforementioned properties with the simplicity of their synthesis and potential recyclability has led to the use of ionic liquids in various extant industrial applications.[21]

One of the most elegant examples of the industrial potential of ionic liquids was the demonstration that some imidazolium-based systems could successfully solubilise *in-situ* cellulose.[21,26-28] This has been suggested to involve disruption of the intermolecular hydrogen-bonding network of cellulose.[27,29-31] More recently, other imidazolium-based ionic liquids have been shown to solubilise suberin isolated enzymatically from potato[32] and to extract lignin from lignocellulosic materials[33]. However, the recalcitrance to biodegradation of the imidazolium moiety,[34] together with its toxicity,[22] may restrict the large-scale application of these interesting observations.

In this context, there is no doubt that one landmark was the demonstration, by our group, that some cholinium alkanoates can efficiently extract *in-situ* suberin from cork.[35] These ionic liquids have also shown to be both benign to eukaryotic organisms and biodegradable.[35,36] However, the detailed chemical and structural characterisation of the extracted suberinic material is yet to be attained. This constitutes the main goal of the present study and a key aspect to understand and improve the efficiency of this process. The suberinic materials isolated with cholinium hexanoate were characterised in terms of chemical composition, morphology, and thermal behaviour. Aiming to better understand the extraction process some alkanoates not considered in the previous study, were also investigated. The extracted suberinic material was found to be mainly composed of oligomeric and polymeric ester type structures.

3. Materials and Methods

3.1 Cork

Granulated cork was obtained from the cork producers Amorim & Irmãos SA (Stª Maria de Lamas, Portugal). The samples were ground to a fine powder (60 mesh) using a centrifuge mill (Retsch) and the cork extractives removed by sequential Soxhlet

extraction with solvents of increasing polarity (dichloromethane, ethanol and water) as previously described by Gil *et al.*[17] The extractives-free cork powder, hereinafter defined solely as cork, was further washed in an excess of deionised water for complete removal of low molecular weight compounds, and then dried prior to use.

3.2 Ionic liquids

The complete list of ionic liquids (Figure 1) used in this study is as follows: 1-ethyl-3-methylimidazolium hexanoate ($[C_2mim][O_2CC_5H_{11}]$); cholinium hexanoate ($[N_{111}C_2H_4OH][O_2CC_5H_{11}]$); cholinium octanoate ($[N_{111}C_2H_4OH][O_2CC_7H_{15}]$) and cholinium decanoate ($[N_{111}C_2H_4OH][O_2CC_9H_{19}]$). The cholinium alkanoate salts were synthesised by dropwise addition of the corresponding acid to aqueous cholinium hydrogencarbonate (Sigma ~80 % in water) in equimolar quantities, as described by Petkovic *et al.*[36] The 1-ethyl-3-methylimidazolium hexanoate was prepared using a similar method. First the chloride anion in 1-ethyl-3-methylimidazolium chloride was exchanged by hydroxide using an ion-exchange column (Amberlite® IRN-78). The resulting 1-ethyl-3-methylimidazolium hydroxide was then neutralised by an equimolar quantity of hexanoic acid. 1-Ethyl-3-methylimidazolium chloride of high purity was purchased from Sigma.

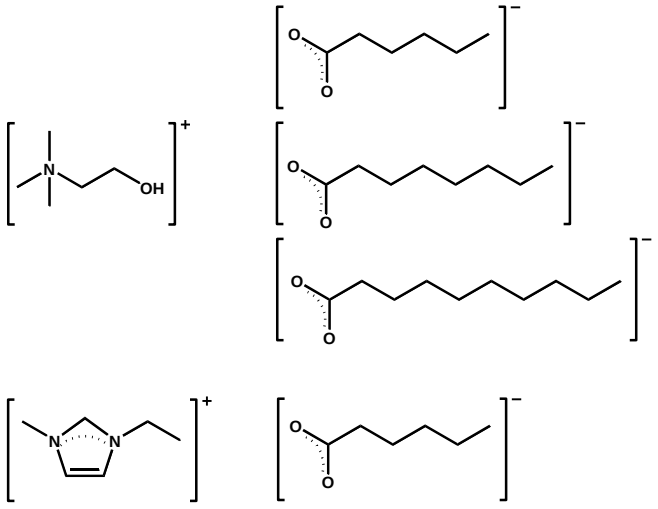


Figure 1| Chemical structures of the tested ionic liquids. From the top: cholinium hexanoate, octanoate and decanoate, and 1-ethyl-3-methylimidazolium hexanoate.

Ionic liquids purity was verified by ^{1}H and ^{13}C NMR spectroscopy at 25 °C, on a Brüker Avance III 400 spectrometer (Brüker BioSpin, Rheinstetten, Germany), and further confirmed by CHNS elemental analysis and electrospray ionisation mass spectrometry (ESI-MS) (Waters LCT Premier fitted with electrospray). The ionic liquids were dried prior to use by stir-heating *in vacuo* (40-70 °C, 24-48 h, *ca.* 0.01 mbar). The water contents, determined by Karl-Fischer titration, were below 0.5 wt %. The obtained salts fulfilled the requirements of the present study.

3.3 Other Chemicals

Ammonium nitrate ($[NH_4][NO_3]$), lithium nitrate ($Li[NO_3]$), dimethyl sulfoxide (DMSO), sodium hydroxide (≥97%), dichloromethane (99%), *n*-hexadecane (99%), decanedioic acid (99%), 12-hydroxydodecanoic acid (≥97%) and deuterated trichloromethane (99.8%) were purchased from Sigma.

3.4 Suberinic material extraction

The experiments followed the protocol previously described by Garcia *et al*.,[35] with some modifications (Figure 2). These aimed exclusively at facilitating and speeding-up the filtration step due to the large amount of suberinic materials being processed. Briefly, the ionic liquid was mixed with powdered cork (ionic liquid : cork ≈ 9 : 1 wt/wt) and kept at 100 °C during 4 h, with stirring (each in triplicate). At the end of the extraction process, DMSO was added to reduce the viscosity of the mixture,[27] facilitating its filtration through a 0.45 µm nylon membrane (Millipore, MA, USA). The cork insoluble residue was then washed thoroughly with an excess of water at 80 °C, and dried at 50 °C under a nitrogen purge until constant weight was attained. The ensuing filtrate, *i.e.* ionic liquid, the extracted material, DMSO and the water added to wash the cork insoluble residue, was kept at 4 °C for 1 h. This led to the precipitation of the extracted suberinic material, which was then recovered by centrifugation (30 min at 4 °C and 2450 *g*), washed twice with an excess of water to remove any remaining ionic liquid, and dried under a nitrogen flux, at 50 °C until constant weight was attained.

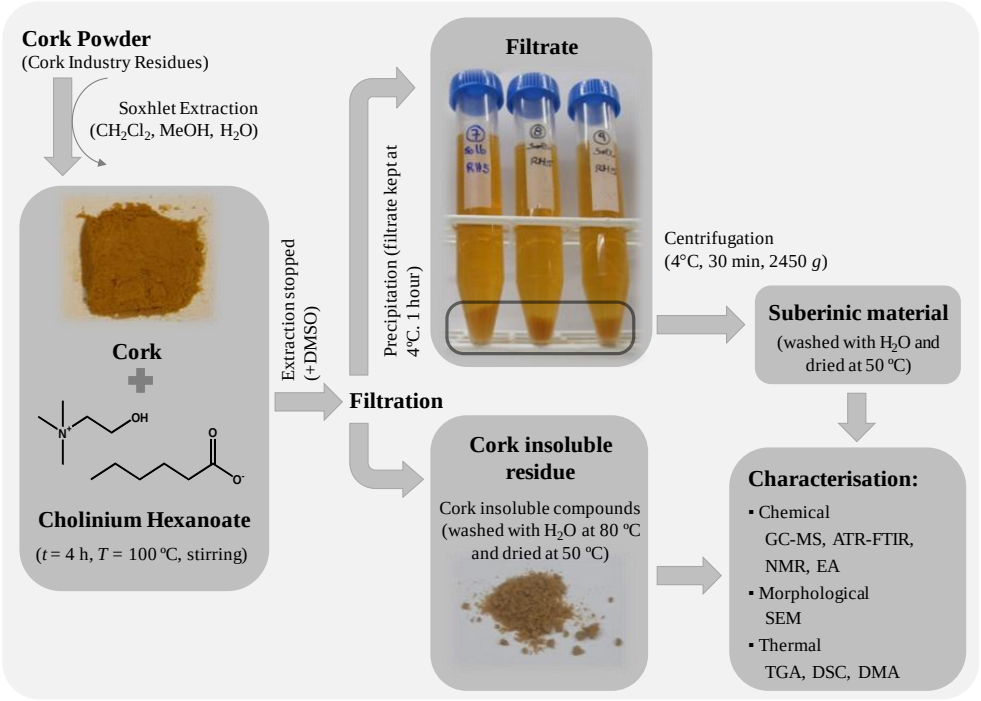


Figure 2| Schematic view of the scientific plan used in this study, where cholinium hexanoate is taken as an example.

3.5 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR spectra were collected on a Brüker IFS66/S FTIR spectrometer (Brüker Daltonics, MA, USA) using a single reflection ATR cell (DuraDisk, equipped with a diamond crystal). Data were recorded at room temperature, in the range of 4000-600 cm^{-1}, by accumulating 258 scans with a resolution of 8 cm^{-1}. Five replica spectra were collected for each sample in order to evaluate reproducibility (OPUS v5.0).

3.6 Scanning Electron Microscopy (SEM)

Samples were dried prior to use and coated with a thin layer of gold using a sputter coater (Polaron E-5100). Electron micrographs were recorded using an analytical field emission gun-scanning electron microscope (FEG-SEM: JEOL 7001F with Oxford light elements EDS detector) operated at 5 - 10 kV. The micrographs presented here were carefully selected, and are regarded to be representative of the different fractions.

3.7 Elemental Analysis (EA)

Elemental composition (C, H and N) was determined using a Leco TruSpec® Series elemental analyser. The oxygen (O) content was assumed to be the remaining amount of the sample and was calculated from the C, H and N composition.

3.8 Nuclear Magnetic Resonance Spectroscopy (NMR)

1D (^{1}H, ^{13}C) and 2D homo- and heteronuclear solution NMR spectra of suberinic materials were acquired on a Avance III 800 spectrometer (Brüker, Rheinstetten, Germany) working at a proton operating frequency of 800.33 MHz, equipped with a three channel 5 mm inverse detection probe head with pulse-field gradients along the *Z* axis. Spectra were run at 25 °C using standard Brüker pulse programs. ^{1}H and ^{13}C chemical shifts are referenced to trichloromethane. ^{13}C spectra were recorded at 201.24 MHz using the APT (attached proton test) sequence. ^{13}C Cross Polarization Magic/Angle Spinning NMR (CP/MAS NMR) spectra were recorded at 9.4 T on a Brüker 400 spectrometer using 9 kHz spinning rate and MAS with proton 90^{o} pulses of 4 μs. Chemical shifts are given in ppm from glycine. All NMR spectra were processed and analysed with MestreNova v. 6.0 (MestreLab Research S.L.).

3.9 Gas Chromatography-Mass Spectrometry (GC-MS)

A Trace GC 2000 Series gas chromatograph equipped with a Thermo Scientific DSQ II mass spectrometer was used. The GC-MS was first calibrated with pure reference compounds (12-hydroxydodecanoic acid and decanedioic acid), representative of the major classes of suberinic compounds, relative to *n*-hexadecane (internal standard). Compounds identification was based on the equipment spectral library (Wiley-Nist) and on previously published data based on their EI-MS fragmentation patterns and/or retention times.[11,13,18,37] Each sample was analysed by two complementary methods:

- Method 1, suberinic material was converted to the corresponding trimethylsilyl derivatives and analysed as previously described,[11] allowing identification of monomeric structures present in the mixture;

- Method 2, in order to analyse the composition of the oligomeric/polymeric fraction of suberinic material, samples were submitted to an alkaline hydrolysis step in order to release their monomeric constituents. Briefly, the samples were treated with a solution of 0.5 M NaOH in methanol/water (1:1, v/v), at 95 °C, during 4 h.[38] The mixture was cooled to room temperature, acidified to pH 3–3.5 with 1 M HCl, extracted three times with dichloromethane, and dried in a rotary evaporator. Finally, samples were trimethylsilylated as mentioned above, prior to GC-MS analysis.

3.10 Thermogravimetric analysis (TGA)

TGA data were obtained using a TGA-Q50 TA Instruments. All samples were run in crimped aluminium pans with pin-hole under a nitrogen atmosphere (100 cm^3 min^{-1}). Samples were dried *in-situ* at 100 °C for 30 min and heated up to 600 °C, at a heating rate of 1 °C min^{-1}. Universal Analysis version 4.4A software was used to determine the degradation temperature ($T_{x\%,\,deg}$), onset temperature (T_{onset}), the weight of water adsorbed by the sample in equilibrium with atmosphere (wt_{H_2O}), the weight of the solid residue remaining at 600 °C ($wt_{600\,°C}$) and the derivative thermograms. $T_{x\%,\,deg}$ and T_{onset} are, respectively, defined as the temperature of a specific weight loss after the drying step, and as the intersection of the baseline weight after the drying step with the tangent of the weight *vs.* temperature curve as decomposition occurs. wt_{H_2O} is defined as the weight loss occurring since the beginning of the experiment until the end of the *in-situ* drying step.

3.11 Differential Scanning Calorimetry (DSC)

DSC analyses were carried out with a DSC – Q200 TA Instrument. The DSC was calibrated for temperature and heat flow with indium samples and operated under constant purging of nitrogen (50 cm^3 min^{-1}). Samples were hermetically sealed in aluminium pans and heated/cooled up to 120/-80 °C at a constant rate of 5 °C min^{-1}, followed by a 5 min isotherm at 120/-80 °C. Three heating/cooling cycles were repeated. The first cycle was used to clear the sample thermal history. When the second and the third cycles were identical, the latter was used for data collection. The characteristic peaks were analysed using Universal Analysis, version 4.4A software. Melting

temperature (T_m) was determined as the minimum of the melting endothermic peak during the heating cycle.

3.12 Dynamic Mechanical Analysis (DMA)

DMA measurements were carried out with Tritec 2000 DMA Triton equipment operating in the bending (single cantilever) mode. Tests were performed at 1 and 10 Hz and the temperature was varied from -100 to 150 ºC at 2 ºC min^{-1}. A small amount of the powdered sample was dispersed in a foldable stainless steel sheet from Materials Pocket of Triton technology.

4. Results and Discussion

Cholinium hexanoate, a biocompatible and biodegradable ionic liquid, was demonstrated to promote a highly efficient extraction of the suberin from cork.[35] These findings were based on analyses of the IR absorption peaks of cork insoluble residues (which can be attributed to its specific constituents without significant error, Supplementary Section S1).[5,6,12,39] The efficiency ranking of the previously tested anions (*viz.* ethanoate < DL-lactate < butanoate ≈ *iso*-butanoate < hexanoate) suggested that the extraction process was controlled by the length of the anion alkylic chain and increases progressively with its basicity.[35] The alkaline requirement of the ionic liquid-based process for the extraction of suberinic materials from cork resembles conventional approaches where this is taken as a critical factor.[5,11,12,16-19,37] In fact, hexanoic acid alone was observed to be unable to extract suberin from cork.[35] In order to mimic the Coulombic component of an ionic liquid environment, eutectic mixtures of inorganic salts ($[NH_4][NO_3]$ + $Li[NO_3]$) were tested. These mixtures also failed to extract cork components (extraction yields < 5 wt %).

4.1 Extraction of suberinic materials from cork with alkanoate-based ionic liquids

Inspired by these initial findings, the extraction of suberinic materials by cholinium alkanoates carrying a long alkylic chain anion and high basicity was tested in the present study. These included the previously tested cholinium hexanoate,[35] and also cholinium

octanoate or decanoate, here tested for the first time. Their extraction ability was initially determined comparing untreated cork with cork treated with each of the ionic liquids, *i.e.* cork insoluble residue (Figure 3). Even though the basicity increases slightly with the length of the anion alkylic chain (hexanoate < octanoate < decanoate), cork mass losses and ATR-FTIR spectral profiles of the cork insoluble residues were comparable (Figure 3a). The ATR-FTIR spectra showed a remarkable reduction of the peak intensities attributed to suberin (2921, 2852, 1737, 1242, 1158 and 724 cm^{-1}). In addition, the polysaccharide and lignin domains in the cork insoluble residues remained apparently unaltered. Data make apparent that the tested cholinium alkanoates led to an extensive extraction of suberin from cork. In view of cork chemical variability, one can assume that the extraction yields obtained in this study, are comparable to the maximum yields reported for alkaline methanolysis of cork (~55 wt %).[12]

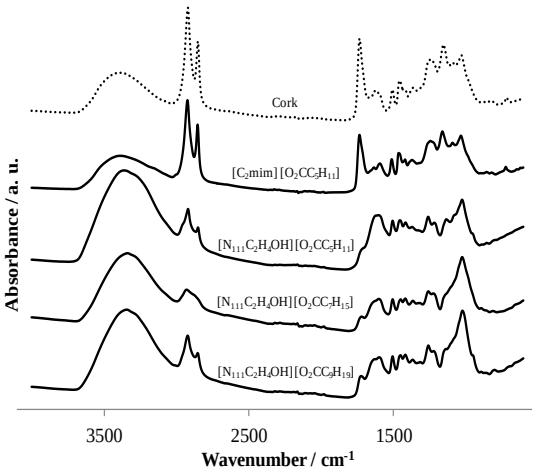
In the present study, it also became apparent that the cholinium cation *per se* plays an important role for suberin extraction. In fact, 1-ethyl-3-methylimidazolium hexanoate was unable to efficiently extract suberin from cork (extraction yields of 30.6 wt %, Figure 3a). The superior performance of the cholinium hexanoate, relative to the 1-ethyl-3-methylimidazolium hexanoate, is probably related with the strength of interaction between cation and anion. In fact, the carboxylate moiety of the anion might strongly interact with the protic hydrogen in the imidazolium ring, partially blocking the extraction of suberin from cork.

Petkovic *et al.* demonstrated that the minimal inhibitory concentration of cholinium hexanoate against fungi was significantly higher (by one order of magnitude) than those of cholinium octanoate or decanoate.[36] It is therefore irrefutable that cholinium hexanoate raises the greenness of this novel suberin extraction process.

4.2 Chemical characterisation of the extracted suberinic material

The superior efficiency of cholinium hexanoate, together with its high biocompatibility,[35,36] makes ultimate its selection for a deeper characterisation of the extracted material, *i.e.* suberinic material.

a)



E_{ext} / %[a]	STD[b]	pK_a (HY)
30.6	2.5	4.85
67.2	2.0	4.85
63.7	1.5	4.89
58.7	6.2	4.90

[a] Extraction yield: E_{ext} / % = 100($m_{cork,\ t=0} - m_{cork\ insoluble\ residue}$) / $m_{cork,\ t=0}$;
[b] STD = standard deviation

b)

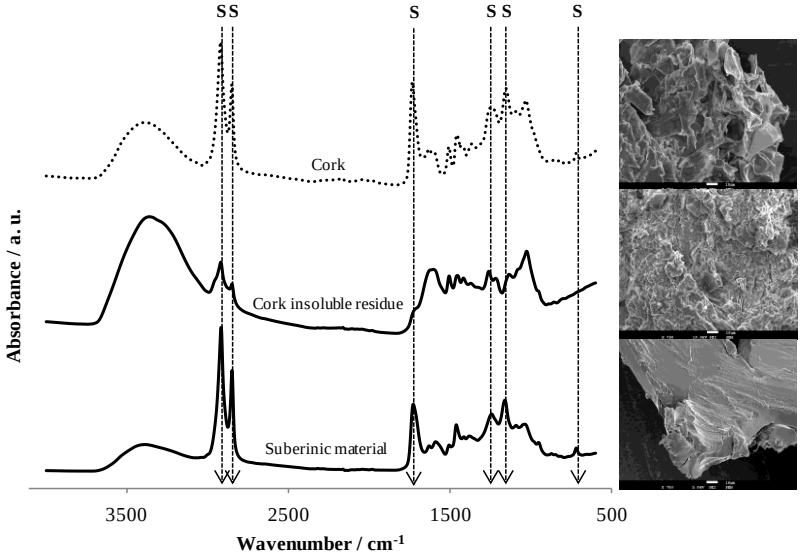


Figure 3| Analysis of the extraction of suberin from cork with the selected ionic liquids, namely 1-ethyl-3-methylimidazolium hexanoate, cholinium hexanoate, cholinium octanoate and cholinium decanoate. (**a**) ATR-FTIR spectra of cork insoluble residue. Side table shows suberin extraction yield after ionic liquid treatment and the pK_a of the conjugate acid (HY) of the corresponding anion. (**b**) ATR-FTIR spectra of the cork insoluble residue and the suberinic material after extraction with cholinium hexanoate. Side figures show the corresponding SEM images (magnification 750×). Vertical lines stand for peaks mainly assigned to suberin (S).

Samples of cork, suberinic material and cork insoluble residue were analysed by SEM (Figure 3b). This provided information on morphological alterations introduced in cork after extraction with cholinium hexanoate. The SEM images show that the cholinium hexanoate extraction process has substantially affected the morphology of powdered cork. A drastic destruction of the cork cell walls can be noticed in the cork insoluble residue. In addition, the suberinic material displayed a very homogenous morphology typical of non-structured material.

Elemental analysis showed that the C, H, O and N relative abundance in the suberinic material (Table 1) were similar to that of a suberin sample extracted through alkaline methanolysis.[13] As expected, due to the presence of long aliphatic chains, the suberinic material was more enriched in C and H when compared to the cork insoluble residue. Likewise, enrichment in hydroxycinnamic acid derivatives, *e.g.* feruloyltyramine,[40] might justify the increment in N relative content detected in the suberinic material. However, vestigial amounts of cholinium hexanoate might also have contributed to this increase both in the suberinic material and in the cork insoluble residue. The increment in the O relative content detected in the cork insoluble residue is most probably due to its enrichment in polysaccharides and lignin.

The ATR-FTIR spectrum of the suberinic material (Figure 3b) is dominated by major peaks at 2921, 2852 cm^{-1}, normally attributed to the long aliphatic chains of suberin.[5,12,13,39] In addition, the high intensity of the band at 1730 cm^{-1}, which is usually assigned to the vibration of carbonyl groups typical of esters, suggests that this material was extracted mainly in the esterified form.

Table 1| Elemental analysis of cork, cork insoluble residue, and suberinic material.

	C / wt %	**H / wt %**	**O / wt %**	**N / wt %**
Cork	61.90	7.41	30.14	0.55
Cork insoluble residue	55.20	6.37	36.17	2.26
Suberinic material	67.40	9.09	22.35	1.16
Suberin[13]	68.00	9.76	20.66	n.d.

n.d. – not determined

The ^{13}C CP/MAS NMR spectrum clearly demonstrates that the suberinic material (Figure 4) owns an essential aliphatic and esterified nature. In fact, the two major resonances at δ 30 and 33 ppm are attributed to methylenic carbons of typical long aliphatic carbon chains; and the resonance at around δ 173 ppm is assigned to carbonyl carbons of ester groups (Figure 4). The resonance at δ 148 ppm is usually assigned to quaternary carbons present in lignin-type structures.[6,17] However, it is difficult to discriminate if these quaternary carbons are the typical aromatic compounds of suberin, lignin or both. Other resonances at about δ 54, 64, 73 ppm and δ 130 ppm were also detected and are assigned to carbons nearby hydroxyl or ester groups and to vinylic carbons, respectively. Though these resonances are typical of suberin, one cannot disregard that they might also be associated with the presence of polysaccharides and lignin, respectively.

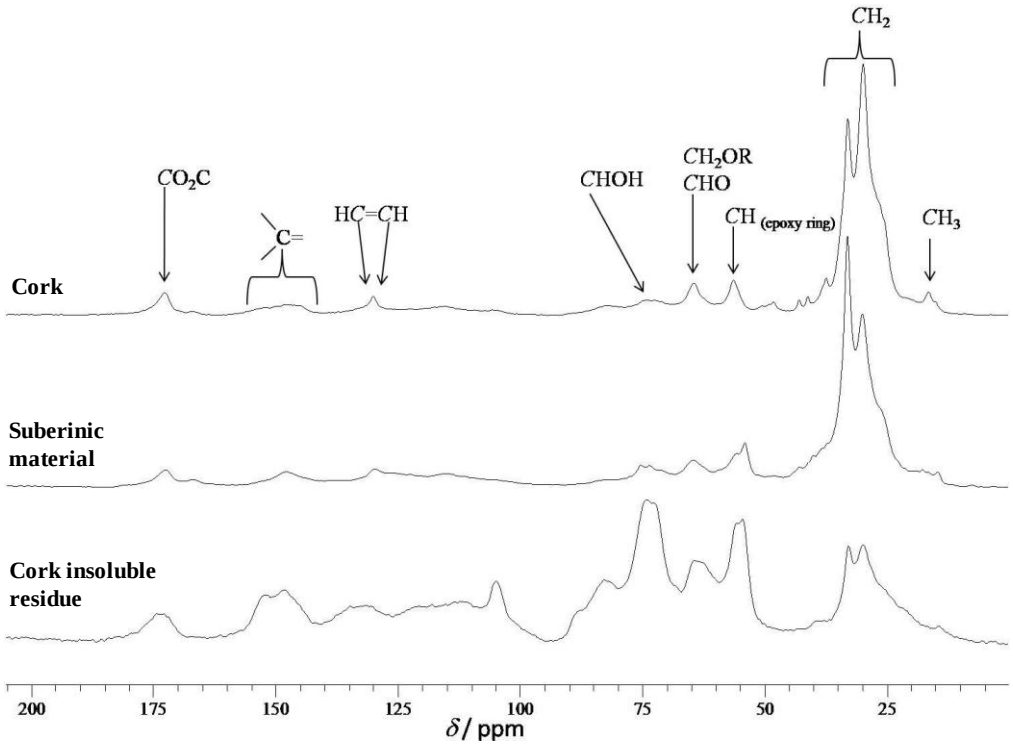


Figure 4| ^{13}C CP/MAS NMR spectra of cork, and both suberinic material and cork insoluble residue after extraction with cholinium hexanoate. R stands for H or ester group.

As expected, the ^{13}C CP/MAS NMR spectrum of the cork insoluble residue showed major resonances typical of polysaccharides and lignin at δ 54, 64, 73, 83, 105 ppm and δ 131-133, 148 ppm, respectively (Figure 4). Although of low intensity, some typical suberin resonances (at δ 30, 33, 173 ppm) were also detected (in accordance with the corresponding ATR-FTIR spectrum, Figure 3b). This seems to imply that suberin extraction from cork by cholinium hexanoate, though extremely efficient, was not complete.

The ^{13}C CP/MAS NMR data further validate the initial interpretation of elemental and ATR-FTIR analyses. The extracted material, which shows an essential aliphatic and esterified nature typical of suberin, could not be completely solubilised in organic solvents. The dichloromethane insoluble cross-linked fraction of the suberinic material represents 42 ± 2 wt %. This observation reinforces that despite the high extraction efficiency of cholinium hexanoate, this process took place by partial depolymerisation of suberin.

4.3 Chemical characterisation of the organic soluble fraction of suberinic materials

In order to complete the chemical characterisation of the suberinic material, the organic soluble fraction was further characterised by ^{1}H and ^{13}C NMR spectroscopy. These methodologies were combined with 2D COSY, HSQC and HMBC studies[41] for refining the spectral attributions (Supplementary Section S2). The data depicted in Table 2 include the list of the functional groups identified and their NMR assignment. The obtained ^{1}H NMR spectrum (Figure 5) is characterised by the presence of a major group of resonances, in the range δ 1.25-2.38 ppm, associated with suberin methylenic groups, in different chemical environments, namely in the long aliphatic chains and nearby ester groups. Two additional resonances at δ 4.05 and 4.82 ppm, assigned to methylenic and methinic protons directly linked to an ester group, were also observed. These features were confirmed by ^{13}C NMR analysis (Table 2) which showed dominant aliphatic carbon resonances at δ 25-35 ppm and at 173-190 ppm assigned to -COO- groups. Other minor resonances were also detected, namely those assigned to vinylic groups (^{1}H: δ 5.34 ppm; ^{13}C: δ 130 ppm), aliphatic methyl groups (^{1}H: δ 0.72-1.05 ppm; ^{13}C: δ 12 ppm) and

aromatic compounds (^{1}H: δ 5.92-8.09 ppm; ^{13}C: δ 100-150 ppm). The presence of aromatic protons (highlighted in the magnified section of Figure 5), confirms also the data observed in the ^{13}C CP/MAS NMR. Overall, the NMR data clearly suggest that the structural features of the suberinic material extracted by cholinium hexanoate are highly consistent with those previously reported for suberin extracted by conventional methods from *Q. suber* cork.[5,11,13,16,17,42] Importantly, using conventional methods only the organic soluble suberinic monomers and oligomers released during hydrolysis are extracted.[7]

Table 2| ^{13}C and ^{1}H NMR analysis assignments of the functional groups identified in the suberinic material.

^{13}C δ / ppm	^{1}H δ / ppm	Functional Group	Assignment[5,11,13,16,17,42]
12	0.72 – 1.05	CH_3	Aliphatic methylic groups
25 – 35	1.25, 1.29	CH_2	Aliphatic methylenic groups
—	1.53 – 1.67	CH_2CH_2CO; CH_2CH_2O	Methylenes in the β position to hydroxylic, ester and carboxylic groups
—	2.00	CH_2=CH-CH	Allylic protons
—	2.25 – 2.38	CH_2COO; CH_2COOH	Methylenes linked to carboxylic moieties
—	3.00	CH	Epoxy ring
62	3.64	CH_2OH; CHOH	Primary and secondary alcohol
52	3.66	OCH_3	Methoxy groups
54	4.05	OCH_2	Methylenes adjacent to ester groups
n.i.	4.82	OCH	Methine adjacent to ester groups
130	5.34	CH=CH	Vinylic groups
100 – 150	5.92 – 8.09	Ar	Aromatic signals
173 – 190	—	COO; COOH	Ester and carboxylic acid groups

n.i – not identified

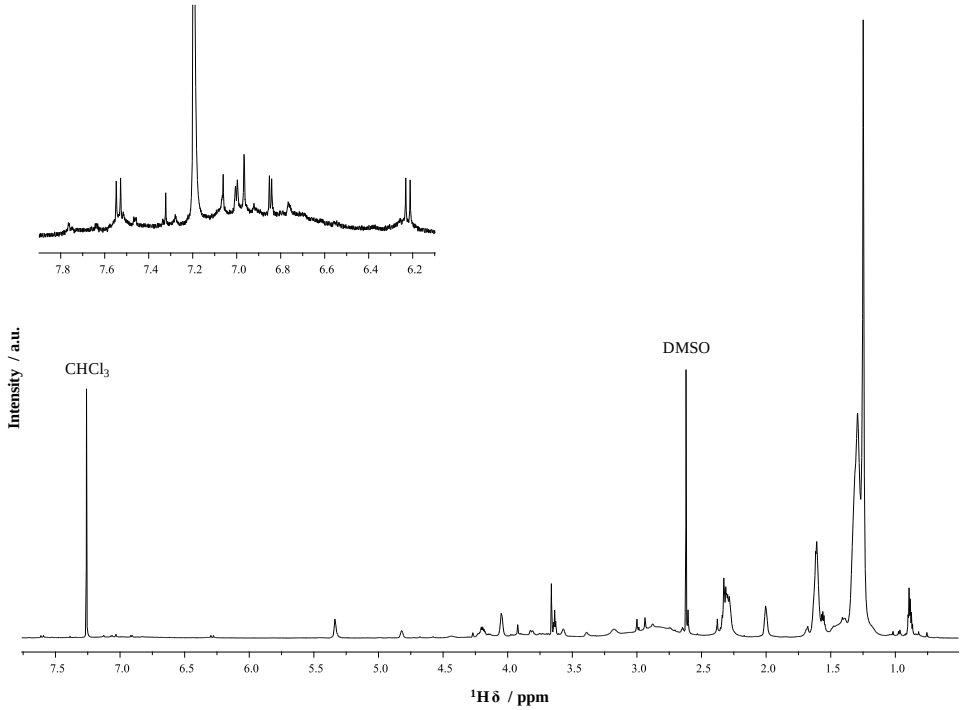


Figure 5| ^{1}H NMR spectrum (800 MHz, 25 °C) of the suberinic material fraction soluble in deuterated trichloromethane. The magnified section of the spectrum corresponds to its aromatic domain.

4.4 Chemical characterisation of the suberinic materials monomers

GC-MS analysis of the monomeric composition of the suberinic material (Method 1) led to the identification of only 3.9 wt % (Table 3). The low identification yield is certainly due to the fact that the suberinic material is mainly in the form of high molecular weight components, *i.e.* oligomeric or polymeric fractions of suberin-type structures. This reinforces the idea of its esterified nature, which is also compatible with the presence of insoluble cross-linked polyester type structures. The main families of monomeric compounds detected were extractives (not covalently bonded to cork), alkanoic acids and monoacylglycerols derivatives, even if each accounted for ≤ 1-2 wt %. Minor amounts of alkan-1-ols, hydroxyacids, alkanedioic acids and aromatic compounds were also identified (< 0.2 wt %).

As reported above, analysis of the suberinic material by GC-MS lead to a very low identification yield of monomers. In order to circumvent this, the GC-MS analysis

was repeated in a sample hydrolysed by alkaline hydrolysis (η = 62 wt %) prior to the silylation (Method 2, Table 3). After hydrolysis, the amounts of detected compounds in the suberinic material were considerably higher, accounting for 36 wt % (~13.5 wt % of cork). These yields are close to those previously reported.[7,13] In the hydrolysed suberinic material, a considerable increment on the content of typical suberin hydroxyacids (*e.g.* 22-hydroxydocosanoic and 9,10,18-trihydroxyoctadecanoic) and alkanedioic acids (*e.g.* 9,10-dihydroxyoctadecanedioic), as well as aromatic compounds (in particular ferulic acid), was observed.[7,11,37] Most monomeric compounds with ≥ 3 OH and/or COOH functionalities were only detected after hydrolysis of the suberinic material. Once more this is in accordance with its cross-linked and therefore insoluble nature.

Table 3| Main suberin monomers identified by GC-MS analysis of the suberinic material by Method 1 and Method 2, *i.e.* non-hydrolysed and hydrolysed samples respectively. Results are given in mg of compound *per* gram of suberinic material.

Identification	Method 1 $m_x/m_{suberin}$ mg/g	Method 2 $m_x/m_{suberin}$ mg/g
Alkan-1-ols	**1.04**	**7,44**
Octadecanol	0.03	0,11
Eicosanol	0.08	0.43
Docosanol	0.64	5.06
Tetracosanol	0.30	1.84
Alkanoic acids	**5.70**	**10.76**
Hexanoic Acid[a]	4.70	1.43
Tetradecanoic acid	0.03	0.24
Hexadecanoic acid	0.24	2.35
Octadeca-9,12-dienoic acid (linoleic acid)	—	0.18
Octadec-9-enoic acid (oleic acid)	0.03	0.21
Octadecanoic acid	0.38	3.16
Eicosanoic acid	0.04	0.12
Docosanoic acid	0.27	3.07
Hydroxyacids	**1.75**	**191.28**
10-Hydroxydecanoic acid	0.05	0.41
16-Hydroxyhexadecanoic acid	—	1.72
18-Hydroxyoctadec-9-enoic acid	0.04	33.83
18-Hydroxyoctadecanoic acid	—	0.79
20-Hydroxyeicos-9-enoic acid	—	1.29
20-Hydroxyeicosanoic acid	0.03	4.11

Table 3| (continued)

Identification	Method 1 $m_x/m_{suberin}$ mg/g	Method 2 $m_x/m_{suberin}$ mg/g
22-Hydroxydocosanoic acid	1.43	68.27
24-Hydroxytetracosanoic acid	0.20	7.94
9,18-Dihydroxy-10-methoxyoctadecanoic acid[b]	—	8.87
9,10,18-Trihydroxyoctadecanoic acid	—	53.00
cis- Mid-chain,18-dihydroxyoctadec-9-enoic acid	—	3.25
trans- Mid -chain,18-dihydroxyoctadec-9-enoic acid	—	2.55
Mid -chain,18-trihydroxyeicosanoic acid	—	5.23
Alkanedioic acids	**0.43**	**49.71**
Hexadecanedioic acid	—	2.48
Octadecanedioic acid	Tr	0.64
Octadec-9-enedioic acid	—	6.32
9,10-Dihydroxyoctadecanedioic acid	—	26.52
Eicosanedioic acid	0.22	1.83
9,10-Dihydroxyeicosanedioic acid	—	3.12
Docosanedioic acid	0.21	8.81
Aromatics	**0.35**	**30.48**
4-Hydroxy-3-methoxybenzaldehyde (vanillin)	Tr	0.65
4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	0.20	1.31
3.4-Dihydroxybenzoic acid	0.08	—
4-Hydroxy-3-methoxy-cinnamic acid (*cis*-ferulic acid)	—	0.84
4-Hydroxy-3-methoxy-cinnamic acid (*trans*-ferulic acid)	0.06	27.68
Extractives	**20.50**	**44.29**
β-Sitosterol	1.47	2.63
Friedelin	9.76	10.14
Betulin	6.95	27.24
Betulinic acid	2.31	4.29
Monoacylglycerol derivatives	**8.57**	**0.00**
1-Monohexadecanoylglycerol	1.30	—
1-Monooctadecanoylglycerol	0.41	—
1-Monodocosanoylglycerol	1.18	—
1-Monotetracosanoylglycerol	1.20	—
1-Mono[docosanedi-22-oic acid-1-oyl]glycerol	4.48	—
Glycerol	**1.92**	**0.43**
Others	**3.18**	**24.90**
Other epoxy derivatives	—	24.90
n.i.	3.18	—
Total Identified Sample (wt %)	**3.87**	**35.79**

tr – trace amounts; n.i. – not identified; [a] Not accounted for compounds quantification; [b]Methoxyhydrin artefact from 9,10-epoxy-18-hydroxyoctadecanoic acid.[13]

4.5 Thermal characterisation of the extracted suberinic material

The thermal characterisation of the suberinic material was performed by TGA (Table 4, Figure 6a) and DSC analyses (Figure 6b). During the TGA analysis the samples were dried *in-situ* showing weight losses (wt_{H_2O}) of 3.02, 5.76 and 2.01 wt % for cork, cork insoluble residue and suberinic material, respectively. Previous studies reported similar values for cork wt_{H_2O}.[43,44]

All the samples were observed to be thermally stable up to approximately 200 °C (Figure 6a). The suberinic material showed the lowest $T_{5\%,\,deg}$. This suggests the presence of a small fraction of volatile molecules, certainly including the suberin free monomeric units detected by GC-MS analysis. The thermal resistance of the suberinic material (T_{onset} = 310.7 °C) was similar to that of cork (T_{onset} = 301.8 °C), and much higher than that of the cork insoluble residue (T_{onset} = 229.6 °C). This agrees with previous reports on similar materials.[45] The close similarity of their decomposition profiles, together with their comparable T_{onset} values, underlines the key role of suberin in cork's high thermal resistance. Accordingly, the cork insoluble residue presented the lowest thermal resistance, certainly owing to its high polysaccharides content, which normally display T_{deg} < 200 °C.[2,39,46] Above 200°C and up to 450 ºC all samples showed a gradual multi-step weight loss, typical of complex biomass-based samples. The solid residue remaining at 600 °C accounted for 20.5, 36.1 and 16.5 wt % for cork, cork insoluble residue and suberinic material, respectively. Even though lower $wt_{600\,°C}$ would be expected for suberinic materials,[13] similar values have been observed in chemically re-polymerised suberin monomers.[47] Hence the high thermal stability of the extracted suberinic material is most probably due to its esterified nature and the presence of cross-linked polyester type structures. In addition, the high value of $wt_{600\,°C}$ for the cork insoluble residue is apparently due to its high content in lignin.[48]

The DSC thermograms of cork and suberinic material, displayed one broad melting transition that span for several tens of degrees (Figure 6b), ranging approximately from 30 to 70 °C. This behaviour is probably a consequence of the complex composition of these samples.

Table 4| Degradation temperature ($T_{x\%,\ deg}$) and onset temperature (T_{onset}). Weight of water adsorbed by the samples in equilibrium with atmosphere (wt_{H_2O}) and weight of the solid residue remaining at 600 °C ($w_{600\ °C}$).

	Cork	Cork insoluble residue	Suberinic material
$T_{5\%,\ deg}$ / °C	231.4	213.0	205.2
$T_{10\%,\ deg}$ / °C	262.6	239.8	257.4
T_{onset} / °C	301.8	229.6	310.7
$wt_{600\ °C}$ / %	20.46	36.12	16.48
wt_{H_2O} / %	3.02	5.76	2.01

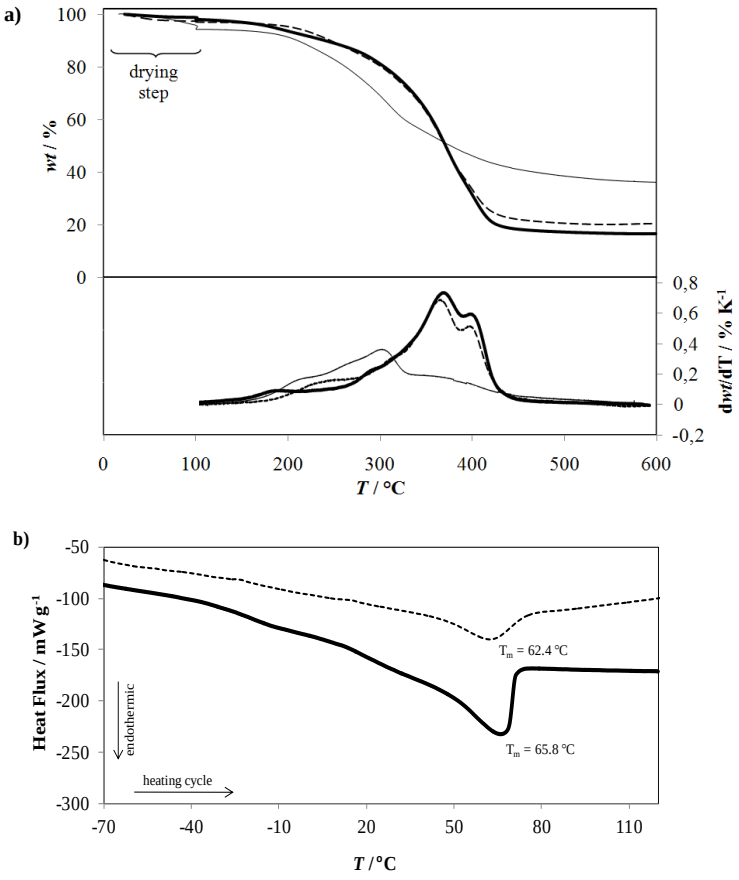


Figure 6| Thermal analyses: (**a**) TGA thermogram (top) and the first derivative of weight loss as a function of temperature (bottom). (**b**) DSC thermogram. (—) cork insoluble residue, (**▬**) suberinic material, (- - -) cork.

A true glass transition for suberin was not observed by DSC, even upon testing different heating/cooling rates (data not shown). Nevertheless, the glass transition for suberin, herein estimated by DMA (which reports higher sensitivity), was -51.0 °C, agreeing with previous reports.[45]

4.6 Environmental sustainability and ionic liquid recyclability

The sustainability of this extraction process can be ensured by optimal design of the filtration step in order to avoid the use of DMSO. Preliminary scale-up tests using cholinium hexanoate were performed (4 h at 100 °C with stirring). At the end of the extraction process, the mixture was immediately filtrated in a pressurised tank at *ca.* 80 °C in order to remove the cork insoluble residue. The ensuing filtrate was then diluted with water and cooled down to 4 °C, leading to precipitation of the extracted suberinic material. The extraction yield and composition (as issued from ATR-FTIR analysis of the materials) were similar to those reported above. The ionic liquid in the aqueous supernatant was recovered by eliminating the water under high vacuum conditions (*ca.* 0.01 mbar). The purity of the recovered ionic liquid was verified by ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry (Supplementary Section S3). The yield of cholinium hexanoate recovered by this method was greater than 99 %. When reused, and accounting for cork chemical variability, no significant loss of efficiency was observed, leading to suberin extraction yield of 58.3 ± 2.3 wt %.

5. Conclusions

The high potential of some cholinium alkanoates, having a long alkylic chain in the anion and high basicity, for extracting suberin from cork was investigated. Cholinium hexanoate showed excellent extraction efficiency and selectivity towards suberin, and high biocompatibility and biodegradability potential. Moreover, it could be easily recycled without loss of extraction efficiency. The chemical and thermal characterisation of the material extracted by cholinium hexanoate, *i.e.* suberinic material, was herein attained for the first time. The extracted material showed suberin typical features, with an aliphatic and esterified nature, and a high thermal resistance. The chemical analysis

before and after alkaline hydrolysis reveals that the isolated suberinic material was mainly composed of cross-linked aliphatic polyester type structures. It seems reasonable to assume that a better understanding of the suberin extraction from cork by cholinium hexanoate, and the detailed study of the structural features of the resulting materials, might hold new insights regarding suberin *in-situ* structural organisation.

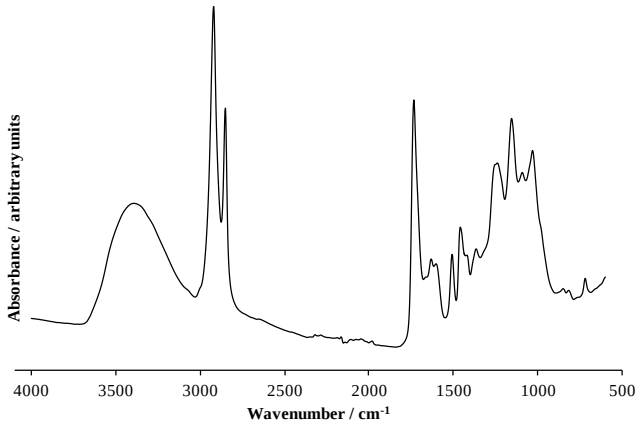
Importantly, this process can be easily applied to other suberin enriched sources, such as birch outer bark. Numerous applications for these new suberinic materials could be envisaged, *e.g.* macromonomers for the synthesis on novel materials.

6. Acknowledgements

R. F. is grateful to *Fundação para a Ciência e a Tecnologia* (FCT), Portugal, for the fellowship SFRH/BD/48286/2008, A. F. S. is also grateful to FCT for the fellowship SFRH/BPD/73383/2010 and H. G. is indebted to *Fundação Calouste Gulbenkian*, Portugal, for the fellowship 21-95587-B. The work was partially supported by a grant from Iceland, Liechtenstein and Norway through the EEA financial mechanism (Project PT015), and FCT through the grants PEst-OE/EQB/LA0004/2011, Pest-C/CTM/LA0011/2011 and PTDC/QUI-QUI/120982/2010. The authors wish to thank I. Nogueira from *Instituto Superior Técnico*, Portugal, for the acquisition of SEM. The National NMR Network (REDE/1517/RMN/2005) was supported by POCI 2010 and FCT, Portugal.

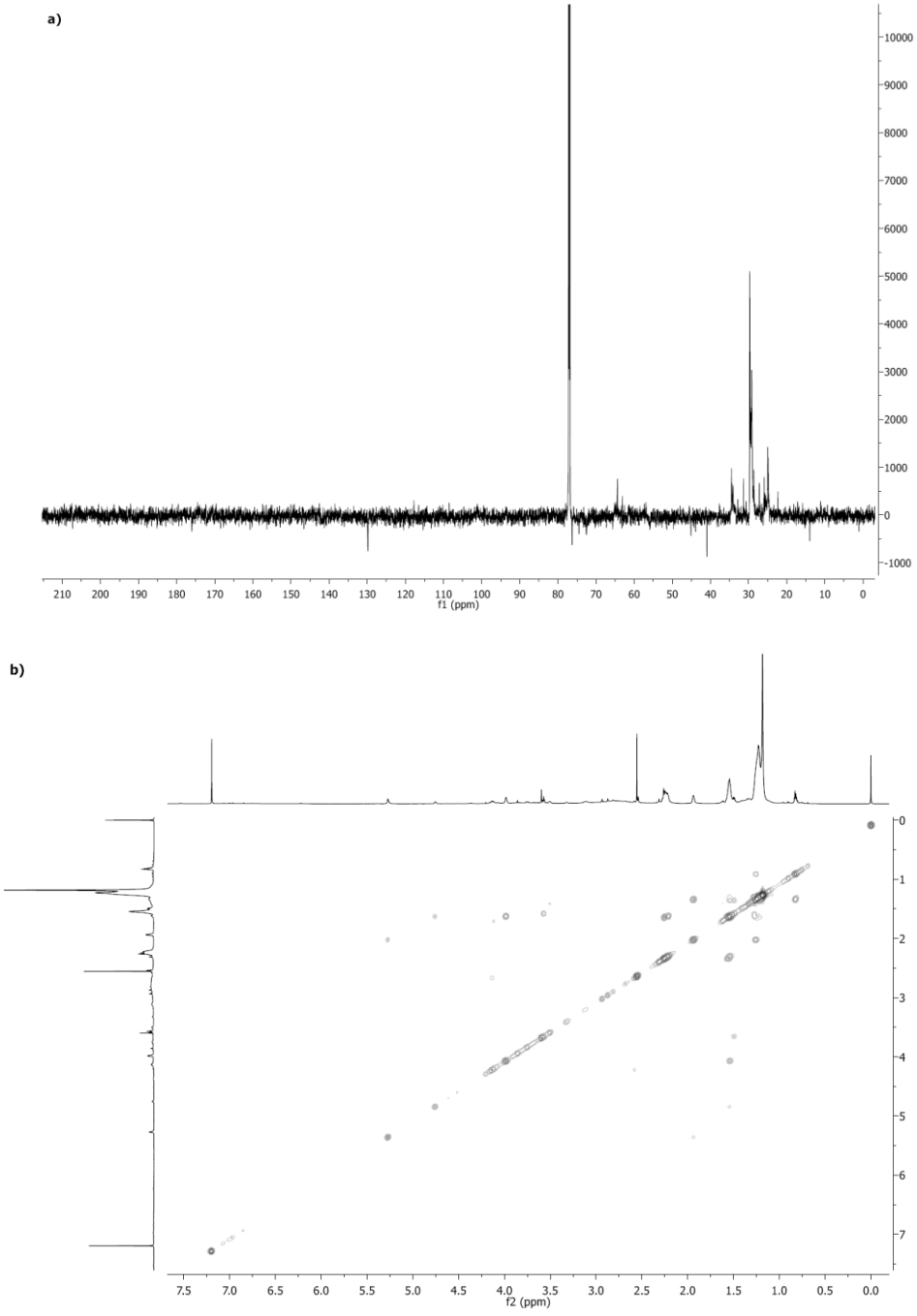
7. Supplementary Information

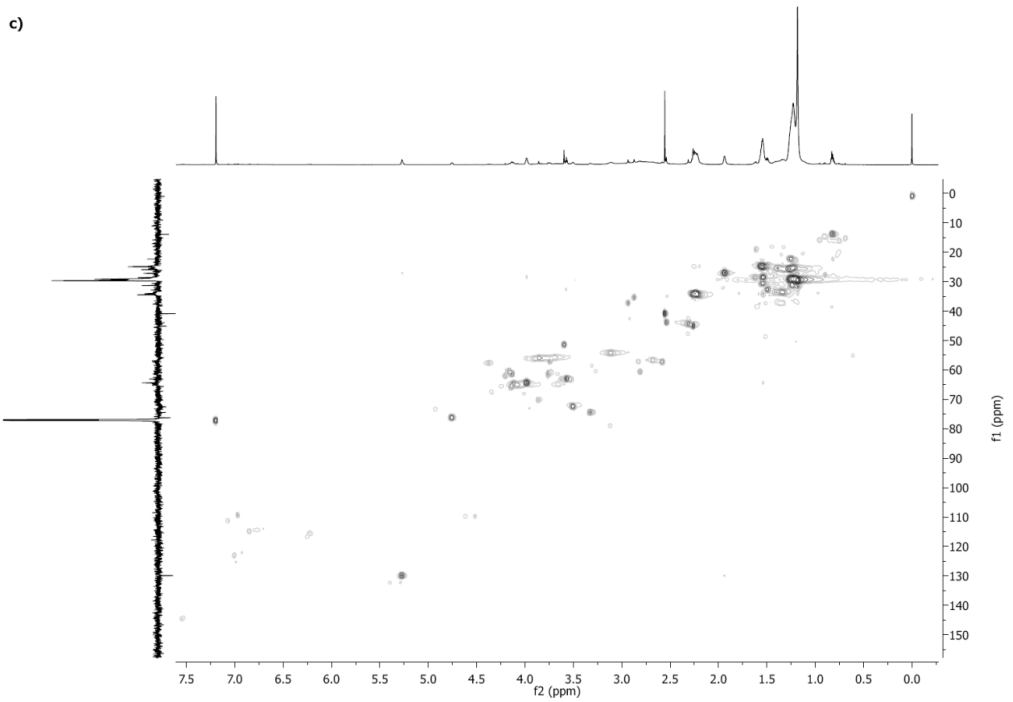
Section 1 (S1)| ATR-FTIR spectrum and assignment of cork major compounds (control),[5,6,12,39] treated under the extracted conditions (4 h at 100 ºC) in the absence of ionic liquid. The underlined cork constituent represents the major contribution to the infrared band.

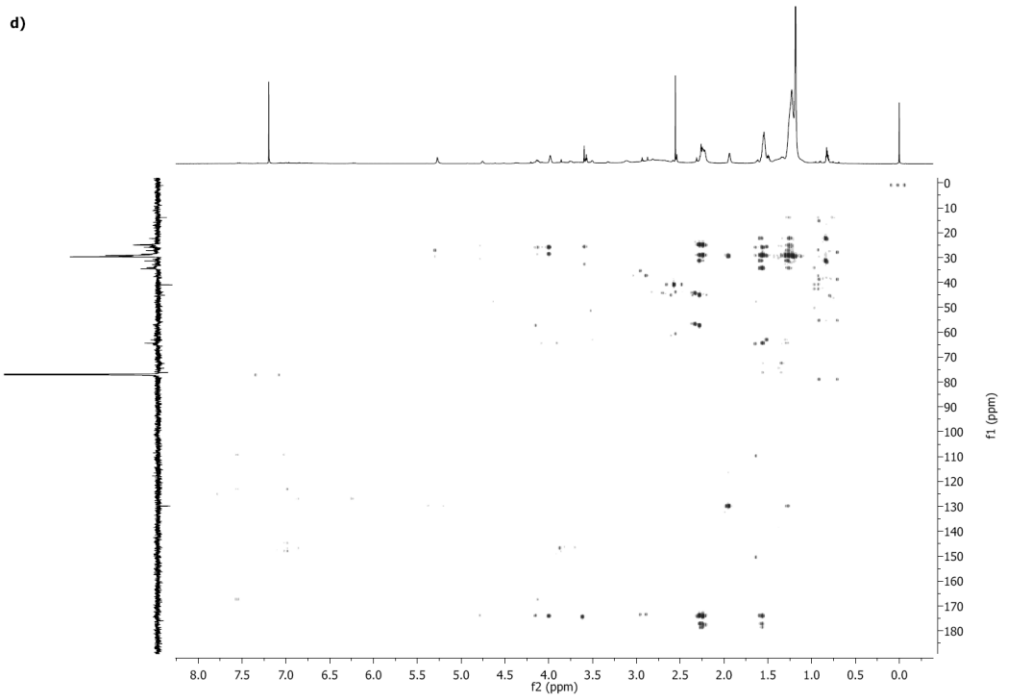


Wavenumber / cm^{-1}	Description	Assignment
3395	OH stretch	water and polissacharides
2921	CH aliph. stretch	suberin, polissacharides and lignin
2852	CH aliph. stretch	suberin, polissacharides and lignin
1737	C=O stretch (ester groups)	suberin, polissacharides and lignin
1635	C=C stretch	suberin
1606	C=C stretch	suberin and aromatic lignin
1511	C=C stretch	aromatic lignin (Guaiacyl-type)
1460	CH asym. deformation	suberin, polissacharides and lignin
1424	C-C stretch in the ring	small amount of aromatic ring Guaiacyl-type lignin
1366	CH sym. deformation	suberin, polissacharides and lignin
1242	CO stretch	suberin, polissacharides and lignin
1158	CO asym. stretch	suberin, polissacharides and lignin
1092	CH, CO deformation	polissacharides and lignin
1034	CH, CO deformation	polissacharides and lignin
855	CH out-of-plane deformation	aromatic ring Guaiacyl-type lignin
819	CH out-of-plane bending	aromatic ring Guaiacyl-type lignin
724	CH_2 rocking	suberin

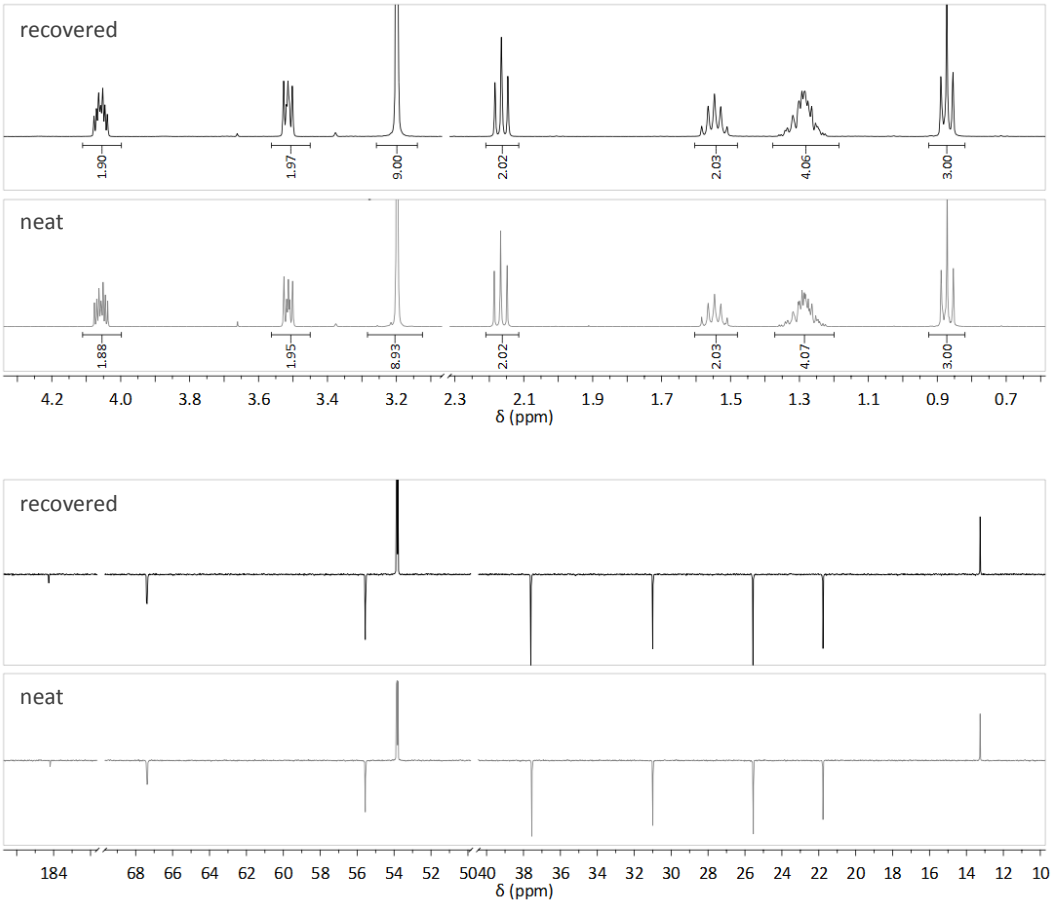
Section 2 (S2)| NMR spectra (800 MHz, 25 °C) of the suberinic material fraction soluble in deuterated trichloromethane: **a**) ^{13}C NMR, **b**) COSY, **c**) HSQC and **d**) HMBC.




c)
f2 (ppm)
f1 (ppm)


d)
f2 (ppm)
f1 (ppm)

Section 3 (S3)| ^{1}H NMR (top) and ^{13}C APT NMR (bottom) spectra of cholinium hexanoate as recovered by water evaporation, compared to those of the neat ionic liquid. Both samples were analysed in D_2O.



ESI-MS recovered cholinium hexanoate:

Calculated for $C_5H_{14}NO^+$ $[M]^+$: m/z = 104.11
Found 104.5
Calculated for $C_6H_{11}O_2^-$ $[M]^-$: m/z = 115.08
Found 114.9

8. References

1. N. Mosier, C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple and M. Ladisch, Features of promising technologies for pretreatment of lignocellulosic biomass, *Bioresour. Technol.*, 2005, **96**, 673-686.
2. S. P. Silva, M. A. Sabino, E. M. Fernandes, V. M. Correlo, L. F. Boesel and R. L. Reis, Cork: properties, capabilities and applications, *Int. Mater. Rev.*, 2005, **50**, 345-365.
3. L. Gil, *Cortiça: produção, tecnologia e aplicação*, INETI, Lisboa, Portugal, 1998.
4. H. Pereira, Chemical composition and variability of cork from *Quercus Suber* L., *Wood Sci. Technol.*, 1988, **22**, 211-218.
5. M. H. Lopes, C. Pascoal Neto, A. S. Barros, D. Rutledge, I. Delgadillo and A. M. Gil, Quantitation of aliphatic suberin in *Quercus suber* L. cork by FTIR spectroscopy and solid-state ^{13}C NMR spectroscopy, *Biopolymers*, 2000, **57**, 344-351.
6. M. H. Lopes, A. S. Barros, C. Pascoal Neto, D. Rutledge, I. Delgadillo and A. M. Gil, Variability of cork from portuguese *Quercus suber* studied by solid-state ^{13}C NMR and FTIR spectroscopies, *Biopolymers*, 2001, **62**, 268-277.
7. A. Gandini, C. Pascoal Neto and A. J. D. Silvestre, Suberin: A promising renewable resource for novel macromolecular materials, *Prog. Polym. Sci.*, 2006, **31**, 878-892.
8. M. A. Bernards, Demystifying suberin, *Can. J. Bot.*, 2002, **80**, 227-240.
9. M. H. Lopes, A. Sarychev, C. Pascoal Neto and A. M. Gil, Spectral editing of ^{13}C CP/MAS NMR spectra of complex systems: Application to the structural characterisation of cork cell walls, *Solid State Nucl. Magn. Reson.*, 2000, **16**, 109-121.
10. R. T. Teixeira and H. Pereira, Suberized cell walls of cork from cork oak differ from other species, *Microsc. Microanal.*, 2010, **16**, 569-575.
11. M. H. Lopes, A. M. Gil, A. J. D. Silvestre and C. Pascoal Neto, Composition of suberin extracted upon gradual alkaline methanolysis of *Quercus suber* L. cork, *J. Agric. Food Chem.*, 2000, **48**, 383-391.
12. J. Graça and H. Pereira, Methanolysis of bark suberins: Analysis of glycerol and acid monomers, *Phytochem. Anal.*, 2000, **11**, 45-51.
13. N. Cordeiro, M. N. Belgacem, A. J. D. Silvestre, C. Pascoal Neto and A. Gandini, Cork suberin as a new source of chemicals: 1. Isolation and chemical characterization of its composition, *Int. J. Biol. Macromol.*, 1998, **22**, 71-80.
14. L. Moire, A. Schmutz, A. Buchala, B. Yan, R. E. Stark and U. Ryser, Glycerol is a suberin monomer. New experimental evidence for an old hypothesis, *Plant Physiol.*, 1999, **119**, 1137-1146.
15. J. Graça, Hydroxycinnamates in suberin formation, *Phytochem. Rev.*, 2010, **9**, 85-91.
16. A. F. Sousa, A. Gandini, A. J. D. Silvestre and C. Pascoal Neto, Determination of the hydroxy and carboxylic acid groups in natural complex mixtures of hydroxy fatty acids by ^{1}H Nuclear Magnetic Resonance spectroscopy, *Appl. Spectrosc.*, 2009, **63**, 873-878.
17. A. M. Gil, M. Lopes, J. Rocha and C. Pascoal Neto, ^{13}C solid state nuclear magnetic resonance spectroscopic study of cork cell wall structure: The effect of suberin removal, *Int. J. Biol. Macromol.*, 1997, **20**, 293-305.
18. R. Ekman and C. Eckerman, Aliphatic carboxylic acids from suberin in birch outer bark by hydrolysis, methanolysis, and alkali fusion, *Pap. Ja Puu-Pap. Timber*, 1985, **67**, 255-273.
19. J. Graça and H. Pereira, Cork suberin: A glyceryl based polyester, *Holzforschung*, 1997, **51**, 225-234.

20. A. Stark and K. R. Seddon, Ionic Liquids, in: *Kirk-Othmer Encyclopedia of Chemical Technology*, ed. A. Seidel, JohnWiley & Sons Inc., Hoboken, New Jersey, USA, 5th edn., 2007, vol. **26**, pp. 836-920.
21. N. V. Plechkova and K. R. Seddon, Applications of ionic liquids in the chemical industry, *Chem. Soc. Rev.*, 2008, **37**, 123-150.
22. M. Petkovic, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Ionic liquids: A pathway to environmental acceptability, *Chem. Soc. Rev.*, 2011, **40**, 1383-1403.
23. M. J. Earle, J. M. S. S. Esperança, M. A. Gilea, J. N. Canongia Lopes, L. P. N. Rebelo, J. W. Magee, K. R. Seddon and J. A. Widegren, The distillation and volatility of ionic liquids, *Nature*, 2006, **439**, 831-834.
24. L. P. N. Rebelo, J. N. C. Canongia Lopes, J. M. S. S. Esperança, H. J. R. Guedes, J. Łachwa, V. Najdanovic-Visak and Z. P. Visak, Accounting for the unique, doubly dual nature of ionic liquids from a molecular thermodynamic, and modeling standpoint, *Acc. Chem. Res.*, 2007, **40**, 1114-1121.
25. K. Shimizu, M. F. Costa Gomes, A. A. H. Pádua, L. P. N. Rebelo and J. N. Canongia Lopes, Three commentaries on the nano-segregated structure of ionic liquids, *J. Mol. Struc-Theochem.*, 2010, **946**, 70-76.
26. D. A. Fort, R. C. Remsing, R. P. Swatloski, P. Moyna, G. Moyna and R. D. Rogers, Can ionic liquids dissolve wood? Processing and analysis of lignocellulosic materials with 1-*n*-butyl-3-methylimidazolium chloride, *Green Chem.*, 2007, **9**, 63-69.
27. N. Sun, M. Rahman, Y. Qin, M. L. Maxim, H. Rodríguez and R. D. Rogers, Complete dissolution and partial delignification of wood in the ionic liquid 1-ethyl-3-methylimidazolium acetate, *Green Chem.*, 2009, **11**, 646-655.
28. H. Ohno and Y. Fukaya, Task specific ionic liquids for cellulose technology, *Chem. Lett.*, 2009, **38**, 2-7.
29. A. Pinkert, K. N. Marsh, S. Pang and M. P. Staiger, Ionic liquids and their interaction with cellulose, *Chem. Rev.*, 2009, **109**, 6712-6728.
30. Y. Fukaya, K. Hayashi, M. Wada and H. Ohno, Cellulose dissolution with polar ionic liquids under mild conditions: Required factors for anions, *Green Chem.*, 2008, **10**, 44-46.
31. I. Kilpeläinen, H. Xie, A. King, M. Granstrom, S. Heikkinen and D. S. Argyropoulos, Dissolution of wood in ionic liquids, *J. Agric. Food Chem.*, 2007, **55**, 9142-9148.
32. M. L. Mattinen, I. Filpponen, R. Järvinen, B. Li, H. Kallio, P. Lehtinen and D. Argyropoulos, Structure of the polyphenolic component of suberin isolated from potato (*Solanum tuberosum* var. Nikola), *J. Agric. Food Chem.*, 2009, **57**, 9747-9753.
33. S. S. Y. Tan, D. R. MacFarlane, J. Upfal, L. A. Edye, W. O. S. Doherty, A. F. Patti, J. M. Pringle and J. L. Scott, Extraction of lignin from lignocellulose at atmospheric pressure using alkylbenzenesulfonate ionic liquid, *Green Chem.*, 2009, **11**, 339-345.
34. S. Stolte, S. Abdulkarim, J. Arning, A.-K. Blomeyer-Nienstedt, U. Bottin-Weber, M. Matzke, J. Ranke, B. Jastorff and J. Thöming, Primary biodegradation of ionic liquid cations, identification of degradation products of 1-methyl-3-octylimidazolium chloride and electrochemical wastewater treatment of poorly biodegradable compounds, *Green Chem.*, 2008, **10**, 214-224.
35. H. Garcia, R. Ferreira, M. Petkovic, J. L. Ferguson, M. C. Leitão, H. Q. N. Gunaratne, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Dissolution of cork biopolymers in biocompatible ionic liquids, *Green Chem.*, 2010, **12**, 367 - 369.
36. M. Petkovic, J. L. Ferguson, H. Q. N. Gunaratne, R. Ferreira, M. C. Leitão, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Novel biocompatible cholinium-based ionic liquids—toxicity and biodegradability, *Green Chem.*, 2010, **12**, 643-649.

37. P. C. R. O. Pinto, A. F. Sousa, A. J. D. Silvestre, C. Pascoal Neto, A. Gandini, C. Eckerman and B. Holmbom, *Quercus suber* and *Betula pendula* outer barks as renewable sources of oleochemicals: A comparative study, *Ind. Crop Prod.*, 2009, **29**, 126-132.
38. A. F. Sousa, P. C. R. O. Pinto, A. J. D. Silvestre and C. Pascoal Neto, Triterpenic and other lipophilic components from industrial cork byproducts, *J. Agric. Food Chem.*, 2006, **54**, 6888-6893.
39. C. Pascoal Neto, J. Rocha, A. Gil, N. Cordeiro, A. P. Esculcas, S. Rocha, I. Delgadillo, J. D. Pedrosa de Jesus and A. J. Ferrer Correia, ^{13}C solid-state nuclear magnetic resonance and *Fourier* transform infrared studies of the thermal decomposition of cork, *Solid State Nucl. Magn. Reson.*, 1995, **4**, 143-151.
40. M. A. Bernards and N. G. Lewis, The macromolecular aromatic domain in suberized tissue: A changing paradigm, *Phytochemistry*, 1998, **47**, 915-933.
41. J. Graça and P. Lamosa, Linear and branched poly(ω-hydroxyacid) esters in plant cutins, *J. Agric. Food Chem.*, 2010, **58**, 9666-9674.
42. N. Cordeiro, M. N. Belgacem, A. Gandini and C. Pascoal Neto, Urethanes and polyurethanes from suberin 2: synthesis and characterization, *Ind. Crop Prod.*, 1999, **10**, 1-10.
43. J. F. Mano, The viscoelastic properties of cork, *J. Mater. Sci.*, 2002, **37**, 257-263.
44. S. Lequin, D. Chassagne, T. Karbowiak, R. Gougeon, L. Brachais and J.-P. Bellat, Adsorption equilibria of water vapor on cork, *J. Agric. Food Chem.*, 2010, **58**, 3438-3445.
45. N. Cordeiro, N. M. Belgacem, A. Gandini and C. Pascoal Neto, Cork suberin as a new source of chemicals: 2. Crystallinity, thermal and rheological properties, *Bioresour. Technol.*, 1998, **63**, 153-158.
46. H. Pereira, The thermochemical degradation of cork, *Wood Sci. Technol.*, 1992, **26**, 259-269.
47. A. F. Sousa, A. Gandini, A. J. D. Silvestre, C. Pascoal Neto, J. J. Cruz-Pinto, C. Eckerman and B. Holmbom, Novel suberin-based biopolyesters: From synthesis to properties, *J. Polym. Sci. Pol. Chem.*, 2011, **49**, 2281-2291.
48. H. Yang, R. Yan, H. Chen, C. Zheng, D. H. Lee and D. T. Liang, In-depth investigation of biomass pyrolysis based on three major components: Hemicellulose, cellulose and lignin, *Energy Fuels*, 2006, **20**, 388-393.

Chapter IV

Isolation of suberin from birch outer bark and cork using ionic liquids

Adapted from: H. Garcia, R. Ferreira, A. F. Sousa, C. S. R. Freire, A. J. D. Silvestre, L. P. N. Rebelo and C. Silva Pereira, Isolation of suberin from birch outer bark and cork using ionic liquids: A new source of macromonomers, *Ind. Crops Prod.*, 2013, **44**, 520–527.

The author had contributed to the planning and execution of all the experiments herein presented, as well as on the discussion, interpretation and preparation of the manuscript. Solid-state NMR and GC-MS analyses were performed in collaboration with technicians or co-authors.

1. Abstract

Cholinium hexanoate, a biocompatible and biodegradable ionic liquid, was recently demonstrated to efficiently and selectively extract suberin from cork, combining high extraction efficiency with isolation of a partial depolymerised material. In the present paper, we report a comparative study of the characterisation of suberin extracted from birch outer bark and from cork using cholinium hexanoate. It became apparent that both extracted suberin samples showed still a cross-linked nature, *i.e.* likely to be closely related to *in-situ* suberin. Suberin samples were mainly constituted by oligomeric or polymeric structures in turn essentially composed of long chain hydroxyacids monomers. Their high thermal stability together with the oligomeric/polymeric nature, open new perspectives for suberin use as macromonomers in the development of bio-based polymeric materials. This also contributes for the valorisation of suberin rich agro-forest residues.

Keywords**:** suberin; cork; birch outer bark; cholinium hexanoate ionic liquids.

2. Introduction

Knowledge on natural polymers, such as starch, cotton, proteins and wool, is ancient and remounts to the beginning of the Human History.[1,2] During the last decade, we have been witnessing a renewed and exponential increase of interest in the production of chemicals, materials, fuels and energy obtained from renewable resources. This is especially true in the so-called biorefinery concept,[3,4] which involves, in many cases, application of valuable components from by-products of agro-forest industries, such as suberin from cork residues.

Suberin, a complex aromatic-aliphatic cross-linked biopolyester, is widespread in the plant Kingdom but it is particularly abundant in *Quercus suber* L. cork (30-50 wt%) and *Betuta pendula* outer bark (40-50 wt %).[5-10] This hydrophobic biopolyester plays a key role as a protective barrier between the plant and the environment.[11] Suberin constitutes a major natural source of valuable compounds such as ω-hydroxyacids, α,ω-

dicarboxylic acids and corresponding *mid*-chain epoxy or dihydroxy derivatives.[5,6] These compounds have attracted considerable attention as building blocks for polymer synthesis.[5,12-14]

Wastes derived from birch kraft pulp mills and cork industries are produced in large amounts, corresponding to ~3.4 wt %[15,16] and ~23 wt %[17,18] of the total production, respectively. Up to present, their exploitation is often limited to burning in biomass boilers to produce energy. However, substantial valorisation can be attained if valuable components are extracted prior to burning.

Suberin can be isolated from cork and birch outer bark residues by a set of well defined depolymerisation methodologies. They normally require harsh chemical processes of ester bond cleavage through alkaline methanolysis with sodium methoxide, or by alkaline hydrolysis.[5,19] Suberin partial depolymerisation can also be achieved using more gentle (though less efficient) extraction processes, *e.g.* calcium oxide methanolysis.[20-23]

Advances in suberin extraction under milder and environmentally benign conditions will certainly foster its wider application. Recently it has been demonstrated that extraction of suberin from cork can also be attained using cholinium hexanoate as solvent.[24,25] This biocompatible and biodegradable ionic liquid,[26] was able to promote a specific and efficient extraction of suberin from cork. The isolated suberin will certainly display distinct properties from those obtained by conventional depolymerisation methods. This observation, together with the environmental sustainability of the ionic liquid extraction process, opens perspectives for new applications, directly or after chemical modification.

The successful extraction of suberin from cork with cholinium hexanoate[24,25] prompted us to isolate also suberin from birch outer bark. Our aim is to carry out a comparative study focussing on the chemical composition and the thermal behaviour of suberin samples isolated from cork and birch outer bark. The data makes apparent the high versatility of the ionic liquid mild extraction process for the isolation of oligomeric/polymeric suberin fractions displaying a thermal behaviour comparable to that of the starting materials.

3. Materials and Methods

3.1 Cork and Birch outer bark samples

Granulated cork was obtained from the cork producers Amorim & Irmãos SA (Stª Maria de Lamas, Portugal). *Betula pendula* outer bark samples were collected from the debarking line at a birch kraft pulp mill in Finland. The industrial birch outer bark was ground in a laboratory mill to pass a 6-mm screen, followed by separation in water into floating outer bark and sedimented inner bark.

Cork and birch outer bark samples were ground to a powder (< 1 mm) using a centrifuge mill (Retsch) and the soluble extractives removed by Soxhlet extraction with solvents of increasing polarity (dichloromethane, ethanol and water) as previously described by Gil *et al.*[27] The extractive-free powders, hereafter defined solely as cork and birch outer bark (starting materials), were further washed in an excess of deionised water, and then dried prior to use.

3.2 Chemicals

Cholinium hexanoate was synthesised by dropwise addition of hexanoic acid to aqueous cholinium hydrogen carbonate (Sigma ~80 % in water) in equimolar quantities, as described by Petkovic *et al.*[26] The ionic liquid was dried prior to use by stir-heating *in vacuo* (40-50 °C, *ca.* 0.01 mbar). Dimethyl sulfoxide (DMSO) of analytical grade was purchased from Sigma.

3.3 Suberin Extraction

The extraction process followed an optimal methodology previously described by Ferreira *et al.*[25] Briefly, the cholinium hexanoate (T_m = 60.57 °C) was mixed with cork or birch outer bark (ionic liquid : powder ≈ 9:1 wt/wt) and kept at 100 °C during 4 h, with stirring. At the end of the extraction process the mixture was filtrated (DMSO was added, 5 – 10 times the reaction volume, in order to assist the filtration step) and the residue washed thoroughly with an excess of water. The filtrate was kept at 4 °C for a period of

1h, leading to the precipitation of the extracted suberin, which was then isolated by centrifugation, washed twice with an excess of water, and dried.

3.4 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR spectra were collected on a Brüker IFS66/S FTIR spectrometer (Brüker Daltonics, MA, USA) using a single reflection ATR cell (DuraDisk, equipped with a diamond crystal). Data were recorded at room temperature, in the range of 4000 - 600 cm^{-1}, by accumulating 128 scans with a resolution of 4 cm^{-1}. Five replica spectra were collected for each sample in order to evaluate reproducibility (OPUS v5.0).

3.5 ^{13}C Cross Polarization / Magic Angle Spinning Nuclear Magnetic Resonance Spectroscopy (^{13}C CP/MAS NMR)

^{13}C CP/MAS NMR spectra were recorded at 9.4 T on a Brüker 400 spectrometer using 9 kHz spinning rate and MAS with proton 90° pulses of 4 μs. Chemical shifts are given in ppm from glycine. The NMR spectra were processed and analysed with MestreNova v. 6.0 (MestreLab Research S.L.).

3.6 Gas Chromatography-Mass Spectrometry (GC-MS)

A Trace GC 2000 Series gas chromatograph equipped with a Thermo Scientific DSQ II mass spectrometer was used. The GC–MS was first calibrated with pure reference compounds (representative of the major classes of compounds present in suberin) relative to *n*-hexadecane (internal standard). Compounds identification was based on the equipment spectral library (Wiley–Nist) and on previously published data, focussing their EI-MS fragmentation patterns and/or retention times.[6,8,19,28] Replicates were done to guarantee low variability and each analysis repeated twice. Each sample was analysed by two complementary methods:

- Method 1, Suberin samples were converted to the corresponding trimethylsilyl (TMS) derivatives and analysed quantitatively by GC–MS, allowing the identification of monomeric structures present in the mixture. In brief, suberin samples (*ca.* 15 mg) were

reacted with 250 μL of pyridine, 250 μL of *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide and 50 μL of trimethylchlorosilane during 30 minutes at 70 °C.[16]

- Method 2, in order to analyse the composition of the oligomeric/polymeric fraction of suberin, samples were, prior to the silylation, submitted to an alkaline hydrolysis to release hydrolysable monomeric constituents. Briefly, suberin samples were treated with a solution of 0.5 M NaOH in methanol/water (1:1, v/v), at 95 °C, during 4h.[29] The mixture was cooled to room temperature, acidified to pH 3–3.5 with 1 M HCl, extracted three times with dichloromethane, and the combined organic extracts were dried in a rotary evaporator. Finally, samples were trimethylsilylated as mentioned above, prior to GC-MS analysis.

3.7 Thermogravimetric analysis (TGA)

TGA data were obtained using a TGA – Q50 TA Instruments. All samples were run in crimped aluminium pans with pin-hole under a nitrogen atmosphere (100 cm^3 min^{-1}). Samples were heated up to 600 °C, at a heating rate of 10 °C min^{-1}. Universal Analysis, version 4.4A software was used to determine: degradation temperature ($T_{x\%,\,deg}$), onset temperature (T_{onset}), maximum decomposition temperature ($T_{d,max}$), weight of water adsorbed by the sample in equilibrium with atmosphere (wt_{H_2O}), weight of the solid residue remaining at 600 °C ($wt_{600\,°C}$) and derivative thermograms (DTGA). $T_{x\%,\,deg}$ and T_{onset} were respectively defined as the temperature of a specific weight loss and as the intersection of the baseline weight with the tangent of the weight *vs.* temperature curve as decomposition occurs. $T_{d,max}$ and wt_{H_2O} were respectively defined as the derivative curve (dwt/dT) maximum and the weight loss occurring since the beginning of the experiment until 100 °C.

3.8 Differential Scanning Calorimetry (DSC)

DSC analyses were carried out with a DSC – Q200 TA Instrument. The DSC was calibrated for temperature and heat flow with indium samples and operated under constant purging of nitrogen (50 cm^3 min^{-1}). Samples were hermetically sealed in aluminium pans and heated/cooled up to 120/-80 °C at a constant rate of 5 °C min^{-1},

followed by a 5 min isotherm at 120/-80 °C. Three heating/cooling cycles were repeated. The first cycle was used to clear the sample thermal history. When the second and the third cycles were identical, the latter was used for data collection. The characteristic peaks were analysed using Universal Analysis, version 4.4A software. Melting temperature (T_m) was determined as the maxima of the melting endotherm peak during the heating of the sample.

4. Results and Discussion

Cholinium hexanoate, a biocompatible and biodegradable ionic liquid,[26] was able to promote a highly efficient extraction of suberin from cork, isolated as a partially depolymerised biopolyester.[24,25] The ionic liquid extraction process was in the present study used for the first time to isolate suberin from birch outer bark. A selective extraction of suberin from birch outer bark was attained; similarly to suberin extraction from cork. The extraction and recovery yields were 57.9 and 37.8 wt %; and 48.4 and 39.9 wt % for cork and birch outer bark, respectively. Cork and birch outer bark display a natural abundance of suberin of 30-50 wt % and 40-50 wt %, respectively.[5-10] However, when accounting the removal of extractives, suberin represents typically 50-60 wt % in the starting materials used here. Therefore, it became obvious that while the extraction yield of the process reveals the natural abundance of suberin in the starting material, the recovery yield points out that some losses occurred during suberin precipitation.

DMSO, which does not affect both the efficiency and selectivity of the process, was used to facilitate the filtration step. The process sustainability can be ensured by proper filtration design.[25]

4.1 Chemical Characterisation of suberin

Suberin samples were characterised using ATR-FTIR, ^{13}C CP/MAS NMR and GC-MS analyses. ATR-FTIR spectra of cork and birch outer bark and the corresponding suberin samples extracted by the ionic liquid are depicted in Figure 1. The spectral profiles of both starting materials (cork and birch outer bark) are very similar, as well as those of both suberin samples, and comparable with previously published data for suberised

tissues.[28] Both suberin samples showed the typical suberin features, highlighted in the spectra by vertical lines (Figure 1). Major peaks at 2920 and 2851 cm^{-1} are mainly attributed to the aliphatic chains of suberin, accounting for asymmetric and symmetric C–H stretching vibrations, respectively. The presence of C=O stretching vibration at 1735 cm^{-1} corresponds essentially to the ester groups in suberin. This peak shows also a shoulder at 1712 cm^{-1} that is usually associated with the C=O group of free carboxylic acids; more perceptible in birch outer bark suberin (Figure 1). The aforementioned perfectly agrees with the observation that suberin was partially depolymerised during the ionic liquid extraction process.[25] In addition, the very broad band between 3679 and

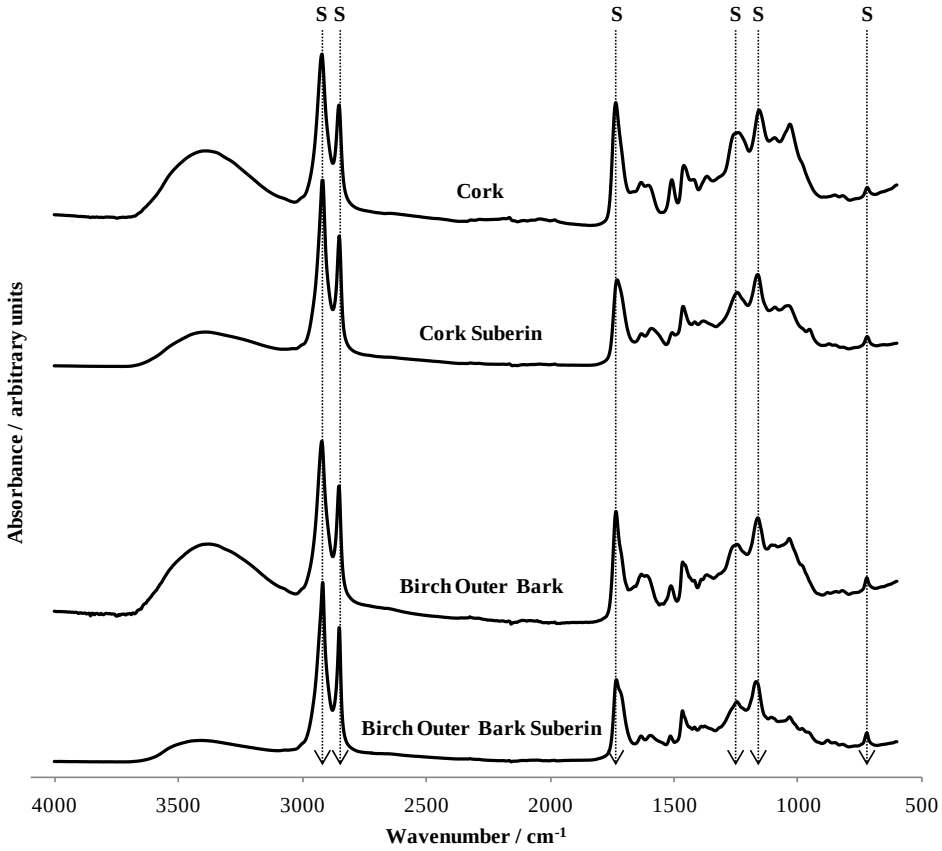


Figure 1| ATR-FTIR spectra of cork and birch outer bark and the corresponding isolated suberin samples following their extraction by cholinium hexanoate. Vertical lines stand for peaks mainly assigned to suberin (S).

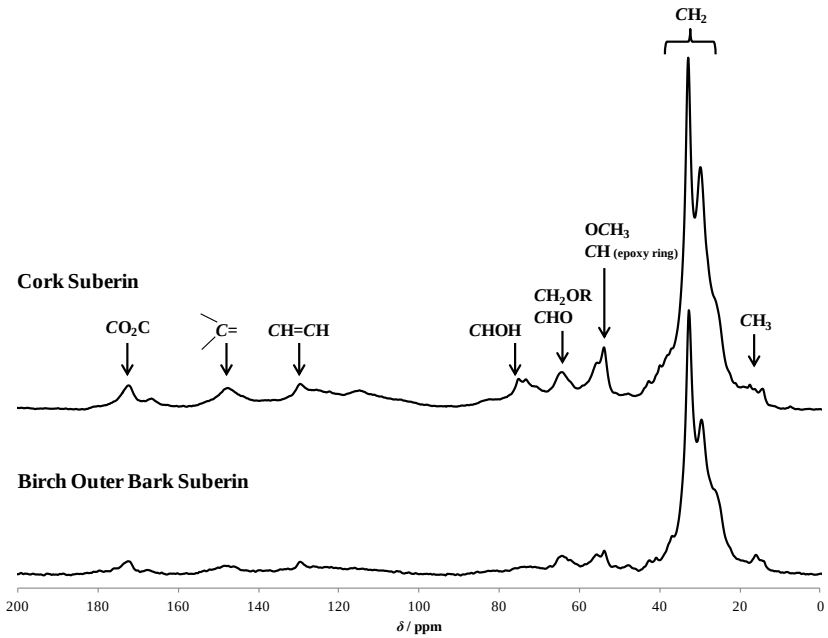


Figure 2| ^{13}C CP/MAS NMR spectra of cork suberin and birch outer bark suberin extracted with cholinium hexanoate. R stands for H or ester group.

3034 cm^{-1} is normally associated with the O–H stretching vibration and can be attributed to carboxylic acids and alcohol groups. Other spectral suberin peaks at 1245, 1164 cm^{-1} and 722 cm^{-1}, corresponding to symmetric and asymmetric C–O stretching and C–H rocking associated with methylenic groups, respectively, were also observed. One cannot disregard the presence of small amounts of lignin (1511, 855 and 819 cm^{-1}) and polysaccharides (1092 and 1034 cm^{-1}).

The ^{13}C CP/MAS NMR spectral information (Figure 2) corroborates the ATR-FTIR data, thus confirming that both extracted suberin samples display fundamentally an aliphatic and esterified nature. The major peaks are assigned in the spectra (Figure 2). Briefly, resonances at δ30/33 ppm and at δ173 ppm are attributed respectively to the methylenic carbons of long aliphatic chains and to carbonyl carbons of ester groups. In both suberin samples the cholinium hexanoate extraction process led to the enrichment of CH_2 groups in the vicinity of oxygen (δ33 ppm), *i.e.* CH_2O groups. A similar effect has been observed in cork suberin extracted using alkaline methanolysis.[8] The resonances at

δ148 ppm are usually assigned to quaternary carbons present in lignin-type structures, which can be associated either to the typical aromatic domains of suberin or lignin.[10,27] Other resonances typical of suberin were also observed, namely δ54, 64, 73 ppm, and δ130 ppm, corresponding to carbons nearby hydroxyl or ester groups and to vinylic carbons, respectively (Figure 2), which can be associated also with the presence of polysaccharides and lignin. The intensities of these peaks, including that at δ148 ppm, were generally higher in the suberin sample isolated from cork relative to that from birch outer bark. Hence it is possible that the natural abundance of lignin and polysaccharides in the starting materials (respectively 25–35 and 15–25 wt % for cork; and 10–15 and 10–15 wt % for birch outer bark)[6,7,9,10] influenced the degree of suberin contamination, if any, with these polymers.

Table 1| Main monomers identified by GC-MS analysis of cork and birch outer bark suberin samples, before and after hydrolysis. Results are given in mg of compound *per* gram of dried starting material.

	Cork Suberin m_x/m_{cork} mg g^{-1}		Birch Outer Bark Suberin $m_x/m_{birch\ bark}$ mg g^{-1}	
Identification (*x*)	**Method 1**	**Method 2**	**Method 1**	**Method 2**
Alkan-1-ols	**0.39**	**2.81**	**0.05**	**0.02**
Octadecanol	0.01	0.04	tr	0.02
Eicosanol	0.03	0.16	—	—
Docosanol	0.24	1.91	0.05	tr
Tetracosanol	0.11	0.70	tr	tr
Alkanoic acids	**2.14**	**4.08**	**0.61**	**8.92**
Hexanoic acid	1.78	0.54	0.24	7.29
Tetradecanoic acid	0.01	0.09	0.02	0.03
Hexadecanoic acid	0.09	0.89	0.13	0.60
Octadeca-9,12-dienoic acid (linoleic acid)	—	0.07	—	0.01
Octadec-9-enoic acid (oleic acid)	0.01	0.08	0.02	0.06
Octadecanoic acid	0.14	1.20	0.06	0.35
Eicosanoic acid	0.01	0.05	0.03	0.06
Docosanoic acid	0.10	1.16	0.11	0.52
Hydroxyacids (hydroxy fatty acids)	**0.66**	**72.30**	**4.33**	**68.20**
10-Hydroxydecanoic acid	0.02	0.15	—	0.17
16-Hydroxyhexadecanoic acid	—	0.65	—	0.31
18-Hydroxyoctadec-9-enoic acid	0.02	12.79	0.16	9.19
18-Hydroxyoctadecanoic acid	—	0.30	0.01	0.21
20-Hydroxyeicos-9-enoic acid	—	0.49	tr	1.49
20-Hydroxyeicosanoic acid	0.01	1.55	0.37	3.82

Table 1| (continued)

	Cork Suberin m_x/m_{cork} mg g^{-1}		Birch Outer Bark Suberin $m_x/m_{birch\ bark}$ mg g^{-1}	
Identification (*x*)	**Method 1**	**Method 2**	**Method 1**	**Method 2**
22-Hydroxydocosanoic acid	0.54	25.81	3.53	16.30
24-Hydroxytetracosanoic acid	0.07	3.00	0.18	0.61
Mid-chain-dihydroxyhexadecanoic acid	—	—	—	2.59
9,18-Dihydroxy-10-methoxyoctadecanoic acid[a]	—	3.35	—	2.41
9,10,18-Trihydroxyoctadecanoic acid	—	20.04	0.08	26.85
cis- *Mid*-chain,18-dihydroxyoctadec-9-enoic acid	—	1.23	—	2.50
trans-*Mid*-chain,18-dihydroxyoctadec-9-enoic acid	—	0.96	—	1.19
Mid-chain-dihydroxyoctadecanoic acid	—	—	—	0.56
Mid-chain, 18-Trihydroxyeicosanoic acid	—	1.98	—	—
α,ω-Diacids	**0.16**	**18.79**	**2.41**	**14.66**
Hexadecanedioic acid	—	0.94	—	0.38
Octadecanedioic acid	tr	0.24	0.08	0.98
Octadec-9-enedioic acid	—	2.39	0.04	4.02
9,10-Dihydroxyoctadecanedioic acid	—	10.02	—	0.93
Eicosanedioic acid	0.08	0.69	0.70	2.34
9,10-Dihydroxyeicosanedioic acid	—	1.18	—	0.16
Docosanedioic acid	0.08	3.33	1.59	5.85
Aromatics	**0.13**	**11.52**	**0.12**	**9.12**
4-Hydroxy-3-methoxybenzaldehyde (vanillin)	tr	0.25	tr	0.06
4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	0.08	0.49	0.06	0.12
3,4-Dihydroxybenzoic acid	0.03	—	—	—
4-Hydroxy-3-methoxy-cinnamic acid (*cis*-ferulic acid)	—	0.32	—	0.17
4-Hydroxy-3-methoxy-cinnamic acid (*trans*-ferulic acid)	0.02	10.46	0.05	8.77
Extractives	**7.75**	**16.74**	**13.06**	**28.78**
β-Sitosterol	0.56	0.99	—	—
Friedelin	3.69	3.83	—	—
Betulin	2.63	10.30	12.28	26.70
Betulinic acid	0.87	1.62	0.77	2.08
Monoacylglycerols Derivatives	**3.23**	**0.00**	**1.80**	**0.00**
1-monohexadecanoylglycerol	0.49	—	0.19	—
1-monooctadecanoylglycerol	0.16	—	0.35	—
1-monodocosanoylglycerol	0.44	—	—	—
1-monotetracosanoylglycerol	0.45	—	—	—
1-mono[docosanedi-22-oic acid-1-oyl]glycerol	1.69	—	1.26	—
Glycerol	**0.73**	**0.16**	**0.54**	**0.15**
Others	**1.20**	**9.41**	**2.63**	**4.07**
other epoxy derivatives	—	9.41	—	4.07
n.i.	1.20	—	2.63	—
Total Identified Sample (wt %)	**1.46**	**13.53**	**2.53**	**12.66**

tr – trace amounts; n.i – not identified. [a] Methoxyhidrin artefact from 9,10-Epoxy-18-hydroxyoctadecanoic acid.[28]

The results of the GC-MS analysis of suberin samples are shown in Table 1 and Figure 3. Direct analysis of suberin samples led to very low GC-MS identification yields (Method 1, see section 3.6), namely 1.5 and 2.5 wt % for cork and birch outer bark, respectively (corresponding to 3.9 and 6.3 wt % of the suberin mass). These results suggest that suberin samples extracted by the ionic liquid were still partially polymerized, or at least in the form of esterified oligomeric structures with molecular weights high enough to hamper their detection by GC-MS. These samples showed also to be largely insoluble in most common organic solvents, *e.g.* the dichloromethane insoluble fraction of suberin extracted from cork and birch outer bark represented 42 ± 2 wt % and 30 ± 3 wt %, respectively. These observations suggest that cholinium hexanoate promotes extraction of suberin with still a polymeric/cross-linked nature, *i.e.* likely to be closely related to *in-situ* suberin.

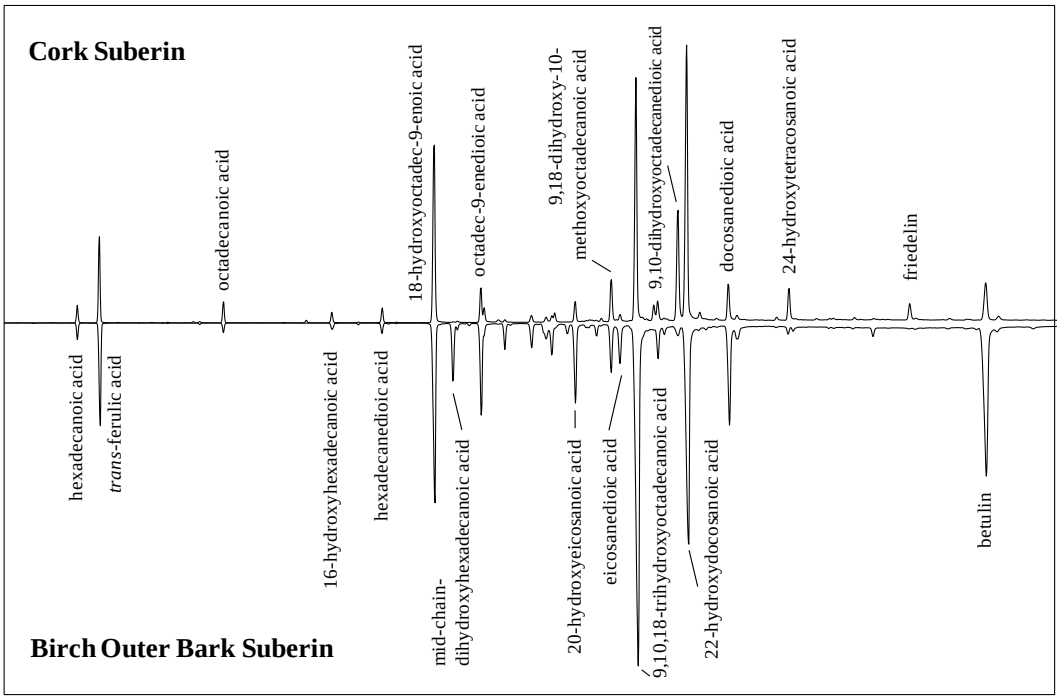


Figure 3| Chromatographic profile of suberin samples extracted from cork and birch outer bark with cholinium hexanoate, as obtained by GC-MS analysis of the hydrolysed samples (Method 2).

In order to circumvent the low GC-MS identification yields, isolated suberin samples were submitted to alkaline hydrolysis (Method 2, see section 3.6) and then analysed by GC-MS (Table 1). The hydrolysed material corresponded to 62.4 wt % and 71.6 wt % of the initial mass of suberin isolated from cork and birch outer bark, respectively. After hydrolysis, the amounts of detected compounds were considerably higher, accounting for approximately 13 wt % of the mass of the starting materials (corresponding to ~35 wt % of suberin mass); in agreement with previously reported results.[5,6,28] The limits in the identification yield are usually attributed to non-volatile high molecular weight fractions.[28]

Both hydrolysed suberin samples reported comparable chemical compositions; as suggested above by the ATR-FTIR and ^{13}C CP/MAS NMR data. These samples showed high abundance of hydroxyacids, α,ω-diacids, extractives, aromatics and alkanoic acids. Other representative compounds, like alkanols and glycerol were also detected in lower amounts. Hydroxyacids detected in cork suberin (72.3 mg g^{-1} of starting material), showed higher abundance of 22-hydroxydocosanoic acid, 9,10,18-trihydroxyoctadecanoic acid followed by 18-hydroxyoctadec-9-enoic acid. On the other hand, in birch outer bark suberin hydroxyacids accounted for 68.2 mg g^{-1} of starting material, mainly composed of 9,10,18-trihydroxyoctadecanoic acid, 22-hydroxydocosanoic acid and 18-hydroxyoctadec-9-enoic acid. Interestingly, both starting materials showed a similar relative abundance of *mid*-chain-hydroxyacids/diacids, some of which might have resulted from ring cleavage of epoxy containing compounds (~40 mg g^{-1} of starting material). This observation differs from previously reported results,[5,6,28] which usually indicate a predominance of epoxy compounds in birch outer bark suberin, especially 9,10-epoxy-18-hydroxyoctadecanoic acid. This dissimilarity can be partially attributed to the selectivity of the extraction process through the use of the ionic liquid. The absence of monomers carrying epoxy rings can also be a consequence of their cleavage, either promoted by the hydrolysis of the suberin samples prior to the GC-MS analyses or by the ionic liquid during suberin extraction. Finally, the relative abundance of aromatics detected by GC-MS in both hydrolysed suberin samples (11.5 and 9.1 mg g^{-1} of cork and birch outer bark, respectively), in particular ferulic acid, reinforces also the findings of ATR-FTIR and ^{13}C CP/MAS NMR analyses.

The GC-MS data confirms that most monomeric compounds detected in suberin, were, prior to hydrolysis, esterified. In addition, these samples also showed some glycerol derivatives, which were not detected in the hydrolysed samples. Glycerol was certainly solubilised in water during the hydrolysis process, as previously reported.[30] In general, the major components detected in the hydrolysed samples are reasonably similar to those regularly detected in suberin samples extracted from cork and birch outer bark by conventional processes.[5,6,8] In addition, most monomeric polyfunctional compounds prone to cross-linking, *i.e.* ≥ 3 OH and/or COOH functionalities, were only detected after hydrolysis of the suberin samples. This strengthens the idea that cholinium hexanoate, regardless of the starting material, extracted suberin owning still a cross-linked nature.

4.2 Thermal Analysis

TGA and DSC analyses were done essentially to evaluate if suberin samples extracted by cholinium hexanoate report a thermal behaviour similar to that of their starting materials.

The TGA thermograms and the most relevant degradation data are shown in Figure 4 and Table 2, respectively. Small weight losses below 100 °C, which are normally associated to water release (wt_{H2O}, Table 2),[31] were detected in all samples. In addition, all the samples were thermally stable up to approximately 200 °C and presented comparable T_{onset}, $T_{5\%wt}$ and $T_{d,max}$ values (Table 2), which highlight the key role of suberin in cork's and birch outer bark's thermal stability. Above 200 °C weight loses were significant and a carbonaceous solid residue was simultaneously formed, which at 600 °C accounted for 10-17 wt % of the initial mass (Table 2).

Table 2| Thermal analyses of cork and birch outer bark and the corresponding suberin samples, namely degradation temperature ($T_{5\%,\ deg}$), onset temperature (T_{onset}), weight of water adsorbed in equilibrium with atmosphere (wt_{H2O}) and weight of the solid residue remaining at 600 °C ($wt_{600\ °C}$).

	Birch Outer Bark Suberin	Birch Outer Bark	Cork Suberin	Cork
$T_{5\%,\ deg}$ / °C	266.8	261.1	232.6	241.5
T_{onset} / °C	369.5	366.3	368.2	348.7
$T_{d,max}$ / °C	422.4	417.1	414.8	407.2
wt_{H2O} / %	0.5	2.1	1.1	2.4
$wt_{600\ °C}$ / %	10.7	15.3	12.9	17.3

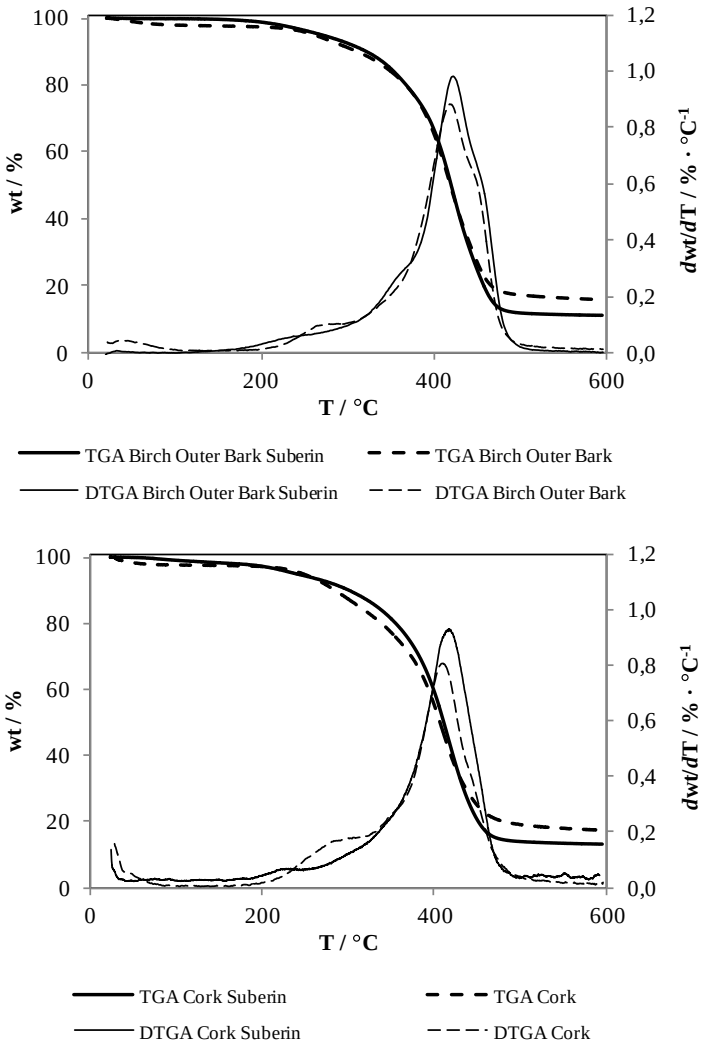


Figure 4| TGA and DTGA curves of cork (bottom) and birch outer bark (top) and the corresponding isolated suberin samples following their extraction by cholinium hexanoate.

The DTGA curves (Figure 4) of the starting materials showed two distinct events during the thermal degradation. The first, a small degradation shoulder occurs at approximately 260-280 °C, which can be mainly attributed to the degradation of hemicelluloses and cellulose.[32,33] This event was more evident in cork due to its higher polysaccharides content. The second event, with a maximum degradation ($T_{d,max}$) in the

range of 400-420 °C corresponds largely to the joint degradation of suberin and lignin.[34,35] Their high thermal stability is due to their *in-situ* three dimensional and heavily cross-linked structures.[36]

In general the TGA curves of both suberin samples were similar to those of the starting materials. Nevertheless the shoulder assigned to polysaccharides in DTGA was much less intense. The TGA data put emphasis on the ionic liquid's high selectivity towards suberin. One cannot disregard however that contamination with minor amounts of lignin and polysaccharides might have occurred.

The DSC thermograms (Figure 5) of cork and birch outer bark and of the corresponding isolated suberin samples showed a broad energetic transition that span for several tens of degrees. This correlates well with the multi-component constitution and the low crystallinity of these samples. Cork and birch outer bark presented a broad endothermic peak on heating at 66.6 and 63.1 °C, respectively, while the corresponding suberin samples showed a similar behaviour, with peaks at 71.2 and 50.3 °C. Accordingly, Cordeiro *et al.*[37] associated the melt-crystallisation cycle of suberin with the presence of a microcrystalline phase ($T_m \approx 10$ - 60 °C).

The thermal behaviour of both suberin samples was in general very similar to that of the starting materials.

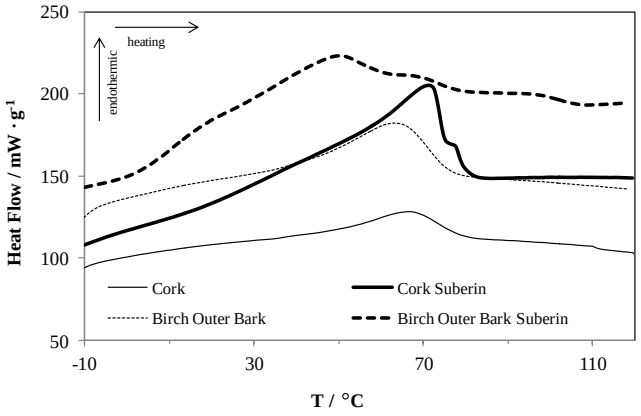


Figure 5| DSC thermograms of cork and birch outer bark and the corresponding isolated suberin samples following their extraction by cholinium hexanoate.

5. Conclusions

The potential of cholinium hexanoate to selectively promote a partial depolymerisation of suberin domains from birch outer bark became evident. This should inspire the application of this extraction process for attempting suberin isolation from other natural sources. Suberin samples extracted from cork and birch outer bark with cholinium hexanoate were observed to be mainly composed of polymeric fractions of suberin-type structures with a comparable thermal behaviour to that of the starting materials. The composing monomeric components were in general similar to those detected in suberin samples obtained by conventional depolymerisation processes. The chemical nature and high thermal stability of the isolated suberin open new perspectives for its use as macromonomers in the development of novel bio-based polymeric materials. Moreover, the extraction of suberin, which is still cross-linked, will certainly provide new opportunities for solving its *in-situ* structure, still under debate.

6. Acknowledgements

R. F. and A. F. S. are grateful to *Fundação para a Ciência e a Tecnologia* (FCT), Portugal, for the fellowships SFRH/BD/48286/2008 and SFRH/BPD/73383/2010, respectively; H. G. is indebted to *Fundação Calouste Gulbenkian*, Portugal, for the fellowship 21-95587-B. The work was partially supported by a grant from Iceland, Liechtenstein and Norway through the EEA financial mechanism (Project PT015), and FCT through the grants PEst-OE/EQB/LA0004/2011, Pest-C/CTM/LA0011/2011 and PTDC/QUI-QUI/120982/2010. The National NMR Network (REDE/1517/RMN/2005), was supported by POCI 2010 and FCT, Portugal. The authors also acknowledge 7th Framework Programme FP7/2007-2013, for funding project AFORE (CP-IP 228589-2), and Thomas Holmbom (SepRes, Finland) for supplying birch outer bark samples.

7. References

1. A. Gandini, The irruption of polymers from renewable resources on the scene of macromolecular science and technology, *Green Chem.*, 2011, **13**, 1061-1083.
2. L. H. Sperling, Introduction to Physical Polymer Science, *John Wiley & Sons Inc.*, New York, 4th edn., 2006.
3. B. Kamm, P. R. Gruber and M. Kamm, Biorefineriess industrial processes and products, *Wiley-VCH*, Weinheim, Germany, 1st edn., 2000.
4. N. Mosier, C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple and M. Ladisch, Features of promising technologies for pretreatment of lignocellulosic biomass, *Bioresour. Technol.*, 2005, **96**, 673-686.
5. A. Gandini, C. Pascoal Neto and A. J. D. Silvestre, Suberin: A promising renewable resource for novel macromolecular materials, *Prog. Polym. Sci.*, 2006, **31**, 878-892.
6. P. C. R. O. Pinto, A. F. Sousa, A. J. D. Silvestre, C. Pascoal Neto, A. Gandini, C. Eckerman and B. Holmbom, *Quercus suber* and *Betula pendula* outer barks as renewable sources of oleochemicals: A comparative study, *Ind. Crops Prod.*, 2009, **29**, 126-132.
7. H. Pereira, Chemical composition and variability of cork from *Quercus suber* L., *Wood Sci. Technol.*, 1988, **22**, 211-218.
8. M. H. Lopes, A. M. Gil, A. J. D. Silvestre and C. Pascoal Neto, Composition of suberin extracted upon gradual alkaline methanolysis of *Quercus suber* L. cork, *J. Agric. Food Chem.*, 2000, **48**, 383-391.
9. M. H. Lopes, C. Pascoal Neto, A. S. Barros, D. Rutledge, I. Delgadillo and A. M. Gil, Quantitation of aliphatic suberin in *Quercus suber* L. cork by FTIR spectroscopy and solid-state ^{13}C NMR spectroscopy, *Biopolymers*, 2000, **57**, 344-351.
10. M. H. Lopes, A. S. Barros, C. Pascoal Neto, D. Rutledge, I. Delgadillo and A. M. Gil, Variability of cork from portuguese *Quercus suber* studied by solid-state ^{13}C NMR and FTIR spectroscopies, *Biopolymers*, 2001, **62**, 268-277.
11. M. Pollard, F. Beisson, Y. Li and J. B. Ohlrogge, Building lipid barriers: biosynthesis of cutin and suberin, *Trends Plant. Sci.*, 2008, **13**, 236-246.
12. A. Olsson, M. Lindström and T. Iversen, Lipase-catalyzed synthesis of an epoxy-functionalized polyester from the suberin monomer *cis*-9,10-epoxy-18-hydroxyoctadecanoic acid, *Biomacromolecules*, 2007, **8**, 757-760.
13. A. F. Sousa, A. Gandini, A. J. D. Silvestre and C. Pascoal Neto, Synthesis and characterization of novel biopolyesters from suberin and model comonomers, *ChemSusChem*, 2008, **1**, 1020-1025.
14. A. F. Sousa, A. Gandini, A. J. D. Silvestre, C. Pascoal Neto, J. J. Cruz-Pinto, C. Eckerman and B. Holmbom, Novel suberin-based biopolyesters: From synthesis to properties, *J. Polym. Sci. Pol. Chem.*, 2011, **49**, 2281-2291.
15. Paper and wood insights, http://www.forestindustries.fi (statistics for 2006), 2006.
16. R. Ekman, The suberin monomers and triterpenoids from the outer bark of *Betula verrucosa* Ehrh., *Holzforschung*, 1983, **37**, 205-211.
17. L. Gil, Cortiça, produção tecnologia e aplicação, *INETI, Lisbon*, 1988.
18. APCOR, Associação Portuguesa de Cortiça, 2009.
19. R. Ekman and C. Eckerman, Aliphatic carboxylic acids from suberin in birch outer bark by hydrolysis, methanolysis, and alkali fusion, *Pap. Puu-Pap. Tim.*, 1985, **67**, 255-273.

20. J. Graça and H. Pereira, Cork suberin: A glyceryl based polyester, *Holzforschung*, 1997, **51**, 225-234.
21. J. Graça and H. Pereira, Glyceryl-acyl and aryl-acyl dimers in *Pseudotsuga menziesii* bark suberin, *Holzforschung*, 1999, **53**, 397-402.
22. J. Graça and H. Pereira, Suberin structure in potato periderm: Glycerol, long-chain monomers, and glyceryl and feruloyl dimers, *J. Agric. Food Chem.*, 2000, **48**, 5476-5483.
23. J. Graça and H. Pereira, Diglycerol alkenedioates in suberin: Building units of a poly(acylglycerol) polyester, *Biomacromolecules*, 2000, **1**, 519-522.
24. H. Garcia, R. Ferreira, M. Petkovic, J. L. Ferguson, M. C. Leitão, H. Q. N. Gunaratne, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Dissolution of cork biopolymers in biocompatible ionic liquids, *Green Chem.*, 2010, **12**, 367-369.
25. R. Ferreira, H. Garcia, A. F. Sousa, M. Petkovic, P. Lamosa, C. S. R. Freire, A. J. D. Silvestre, L. P. N. Rebelo and C. Silva Pereira, Suberin isolation process from cork using ionic liquids: Characterisation of ensuing products, *New J. Chem.*, 2012, **36**, 2014-2024.
26. M. Petkovic, J. L. Ferguson, H. Q. N. Gunaratne, R. Ferreira, M. C. Leitão, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Novel biocompatible cholinium-based ionic liquids—toxicity and biodegradability, *Green Chem.*, 2010, **12**, 643-649
27. A. M. Gil, M. Lopes, J. Rocha and C. Pascoal Neto, A ^{13}C solid state nuclear magnetic resonance spectroscopic study of cork cell wall structure: The effect of suberin removal, *Int. J. Biol. Macromol.*, 1997, **20**, 293-305.
28. N. Cordeiro, M. N. Belgacem, A. J. D. Silvestre, C. Pascoal Neto and A. Gandini, Cork suberin as a new source of chemicals: 1. Isolation and chemical characterization of its composition, *Int. J. Biol. Macromol.*, 1998, **22**, 71-80.
29. A. F. Sousa, P. C. R. O. Pinto, A. J. D. Silvestre and C. Pascoal Neto, Triterpenic and other lipophilic components from industrial cork byproducts, *J. Agric. Food Chem.*, 2006, **54**, 6888-6893.
30. J. Graça and H. Pereira, Methanolysis of bark suberins: Analysis of glycerol and acid monomers, *Phytochem. Anal.*, 2000, **11**, 45-51.
31. S. Lequin, D. Chassagne, T. Karbowiak, R. Gougeon, L. Brachais and J.-P. Bellat, Adsorption equilibria of water vapor on cork, *J. Agric. Food Chem.*, 2010, **58**, 3438-3445.
32. F. Yao, Q. Wu, Y. Lei, W. Guo and Y. Xu, Thermal decomposition kinetics of natural fibers: Activation energy with dynamic thermogravimetric analysis, *Polym. Degrad. Stab.*, 2008, **93**, 90-98.
33. T. Nguyen, E. Zavarin and E. M. Barrall, Thermal analysis of lignocellulosic materials. Part I. Unmodified materials, *J. Macromol. Sci. Rev. Macromol. Chem. Phys.*, 1981, **C20**, 1-65.
34. C. Pascoal Neto, J. Rocha, A. Gil, N. Cordeiro, A. P. Esculcas, S. Rocha, I. Delgadillo, J. D. Pedrosa de Jesus and A. J. Ferrer Correia, ^{13}C solid-state nuclear magnetic resonance and *Fourier* transform infrared studies of the thermal decomposition of cork, *Solid State Nucl. Magn. Reson.*, 1995, **4**, 143-151.
35. H. Pereira, The thermochemical degradation of cork, *Wood Sci. Technol.*, 1992, **26**, 259-269.
36. H. Yang, R. Yan, T. Chin, D. T. Liang, H. Chen and C. Zheng, Thermogravimetric analysis-*Fourier* transform infrared analysis of palm oil waste pyrolysis, *Energ. Fuel*, 2004, **18**, 1814-1821.
37. N. Cordeiro, N. M. Belgacem, A. Gandini and C. P. Neto, Cork suberin as a new source of chemicals: 2. Crystallinity, thermal and rheological properties, *Bioresour. Technol.*, 1998, **63**, 153-158.

Chapter V

Unveiling the dual role of cholinium hexanoate as solvent and catalyst in suberin depolymerisation

Adapted from: R. Ferreira, H. Garcia, A. F. Sousa, M. Guerreiro, F. J. S. Duarte, C. S. R. Freire, M. J. Calhorda, A. J. D. Silvestre, W. Kunz, L. P. N. Rebelo and C. Silva Pereira, Unveiling the dual role of the cholinium hexanoate ionic liquid as solvent and catalyst in suberin depolymerisation, *submitted manuscript*, 2013.

The author had contributed to the planning and execution of most of the experiments herein presented, as well as on the discussion, interpretation and preparation of the manuscript. Solid-state NMR, GC-MS, HPLC and computational analyses were performed in collaboration with technicians or co-authors.

1. Abstract

Disruption of the three-dimensional network of suberin in cork by cholinium hexanoate leads to its efficient and selective isolation. The reaction mechanism, which likely involves selective cleavage of some inter-monomeric bonds in suberin, was still unanswered. To address this question, the role of the ionic liquid during suberin depolymerisation and during cleavage of standard compounds carrying key chemical functionalities was herein investigated. A clear demonstration that the ionic liquid catalyses the hydrolysis of acylglycerol ester bonds was attained herein, both experimentally and computationally (DFT calculations). This behaviour is related to cholinium hexanoate capacity to activate the nucleophilic attack of water. The data showed also that the most favourable reaction is the hydrolysis of acylglycerol ester bonds, with the C2 position reporting the faster kinetics, whilst most of the linear aliphatic esters remained intact. The study emphasises that the ionic liquid plays the dual role of solvent and catalyst and leads to suberin efficient extraction through a mild depolymerisation. It is also one of the few reports of ionic liquids as efficient catalysts in the hydrolysis of esters.

Keywords: Suberin, cholinium hexanoate, ionic liquid, biopolymer, renewable resources, catalyst, hydrolysis, depolymerisation, DFT calculations.

2. Introduction

Renewable resources are increasingly regarded as substituents for highly demanded petroleum-based chemicals.[1] In this context, the extraction, application and biosynthesis of plant biopolyesters, namely cutin and suberin, have been extensively studied.[2-9] Suberin is ubiquitous in higher plants, and particularly abundant in the outer barks of *Quercus suber* and *Betula pendula* and in the peel of *Solanum tuberosum.* This hydrophobic polymeric material is deposited in the secondary cell wall in the internal and the peripheral dermal tissues during cell wall differentiation or as a response to stress and

wounding. Suberin builds an apoplastic barrier that controls the flow of water, gases and ions and protects against biological pathogens and physical aggressions.[9-11]

Since the earliest descriptions, suberin is known to be composed of aromatic and aliphatic monomeric units.[12-14] Currently, the prevailing idea is that suberin comprises two covalently linked domains, the major being the polyaliphatic domain and the minor the polyphenolic one.[10,15-18] Suberin is generally accepted to be organised *in-situ* as a lamellar type structure,[16-19] notwithstanding that some structural aspects are still unanswered. The polyphenolic domain is ingrained on the inner face of the primary cell wall.[10,16-18,20,21] Especially due to its high recalcitrancy, the compositional structure of this lignin-like domain is not yet fully understood.[22,23] Even so, it is known to be composed mainly by hydroxycinnamic acids and their derivatives (predominantly ferulic acid) and some vestigial amounts of monolignols (*viz.* p-coumaryl, coniferyl and sinapyl alcohols). Extensive cross-linking between these aromatic monomers in the lignin-like structure, as well as to other cell wall constituents, is done *via* stable carbon-carbon, amide and ether bonds.[9,23-25]

The polyaliphatic domain is composed mostly of long chains (C_{16}–C_{26}) of alkanols, alkanoic acids, ω-hydroxyalkanoic acids and α,ω-alkanedioic acids (and the corresponding *mid*-chain unsaturated, epoxy or *vic*-diol derivatives) and glycerol. In addition, the deposition of hydroxycinnamates leads to the typical lamellar organisation of suberin of alternate aliphatic and phenolic components.[16] These monomers are in a parallel alignment and linked *via* linear aliphatic ester or acylglycerol ester bonds. Glycerol is a key cross-linker in the formation of a three-dimensional network, connecting hydrophilic moieties and both suberin domains.[16,25]

Knowledge on suberin is spread in diverse scientific disciplines, from biology, *e.g.* the understanding of the polymer biosynthesis,[4,7] to chemistry, *e.g.* the depolymerisation and characterisation of suberin[17]. Although only modestly exploited so far, suberin has been regarded as a source of monomers and oligomers for the synthesis of novel macromolecular materials.[9,26-29] Of particular relevance is the abundance of *mid*-chain hydroxy and epoxy fatty acids in suberin. These are rare in other renewable resources and difficult to synthesise chemically.

Suberin extraction from renewable resources is conventionally attained using alkaline hydrolysis[30] or alkaline methanolysis[17,31]. These methods result in extensive ester bond cleavage, *i.e.* depolymerisation. Although with very low extraction efficiency, comparable methods can be used to promote a partial depolymerisation.[17] An alternative extraction method, using cholinium hexanoate, promotes efficient extraction of a still partially cross-linked and highly polymerised suberin, regardless of the renewable resource used, *viz.* cork[32-34], birch outer bark[34] and potato peel (unpublished work). The uniqueness of such method is highlighted by the compositional structure of suberin, hypothesised to be intimately related to that of the *in-situ* suberin.[32] Emphasis should be also given to the high biocompatibility and biodegradability of the ionic liquid used in the extraction,[35] which can be recycled and reused throughout the process.[32]

Suberin depolymerisation induced by cholinium hexanoate is likely to involve the selective cleavage of some bonds linking suberin composing monomers. This is the underlying question of the present study: to solve the chemical reaction mechanism behind suberin depolymerisation in cholinium hexanoate media. To address this question, we further investigated the lability of key chemical functionalities of suberin and of standard compounds in the ionic liquid media. The chemical mechanism proposed herein was also supported by DFT calculations.

3. Experimental

3.1 Cork

Granulated cork was obtained from the cork producers Amorim & Irmãos SA (Santa Maria de Lamas, Portugal). The samples were ground to a fine powder (60 mesh) using a centrifuge mill (Retsch) and the cork extractives removed by sequential Soxhlet extraction with solvents of increasing polarity (dichloromethane, ethanol and water) as previously described by Gil *et al.*[36]. The extractive-free cork powder, hereinafter defined solely as cork, was further washed with an excess of deionised water for complete removal of low molecular weight compounds, and dried prior to use.

3.2 Chemicals

Cholinium hexanoate was synthesised by dropwise addition of hexanoic acid to aqueous cholinium hydrogen carbonate (Sigma ~80% in water) in equimolar quantities, as previously described.[35] The ionic liquid purity was verified by ^{1}H- and ^{13}C- NMR, CHNS elemental analysis and electrospray ionisation mass spectrometry (ESI-MS). The ionic liquid was dried prior to use by stir-heating in vacuum (40-50 °C, *ca.* 0.01 mbar). The water content, determined by Karl-Fischer titration, was *ca*. 0.2 wt%.

Poly(12-hydroxydodecanedioic acid) was prepared as described before[29] and 9-10-epoxy-18-hydroxyoctadecanoic acid was extracted from *Betula pendula*[37].

Octyl octanoate (≥98%,), glyceryl trioctanoate (≥99%), dimethyl sulfoxide (DMSO, 99.5%), sodium hydroxide (>97%) and dichloromethane (≥99.5%) were purchased from Sigma; glycerol (≥99.5%) from VWR, L-lactic acid (99.5%,) from Fluka and Poly(lactic acid) with an L:D ratio of 96:4 and an M_w of 110000 g mol^{-1} from Cargill-Dow Polymers.

3.3 Suberin extraction

The suberin extraction process followed a methodology which has been previously described.[32,34] Briefly, the cholinium hexanoate (melting temperature, 60.57 °C) was mixed with cork (ionic liquid : cork ≈ 9 : 1 wt/wt) and kept at 100 °C during 1, 2, 4 or 8 hours, with stirring (each in triplicate). At the end of the extraction process, DMSO was added to reduce the viscosity of the mixture,[32] facilitating its filtration through a nylon membrane with an exclusion pore size of 1.0 µm (Millipore, MA, USA). The insoluble residue was then washed thoroughly with an excess of water at 80 °C. Precipitation of the extracted suberin was obtained by keeping the ensuing filtrate (*i.e.* ionic liquid, suberin, DMSO and the water added to wash the insoluble residue) at 4 °C for 1 hour. Suberin was then recovered by centrifugation (30 min at 4 °C and 2450 *g*), washed twice with an excess of water and dried at 50 °C, until constant weight was attained. The aqueous phase (supernatant) was also concentrated in a rotary evaporator and its content in glycerol analysed by high performance liquid chromatography (HPLC).

3.4 Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR spectra were collected on a Bruker IFS66/S FTIR spectrometer (Bruker Daltonics, MA, USA) using a single reflection ATR cell (DuraDisk, equipped with a diamond crystal). Data were recorded at room temperature, in the range of 4000-600 cm^{-1}, by accumulating 128 scans with a resolution of 4 cm^{-1}. Five replica spectra were collected for each sample in order to evaluate reproducibility (OPUS v6.0).

3.5 Nuclear magnetic resonance spectroscopy (NMR)

^{1}H- and ^{13}C-NMR analyses were recorded with a Bruker Avance 400 Ultrashield Plus spectrometer. Spectra were run at 25 °C using standard Bruker pulse programs. ^{13}C CP/MAS NMR spectra were recorded at 9.4 T on a Bruker 400 spectrometer using 9 kHz spinning rate and MAS with proton 90° pulses of 4 µs. Chemical shifts are given in ppm from glycine. The NMR spectra were processed and analysed with MestreNova v6.0 (MestreLab Research S.L.).

3.6 Gas chromatography–mass spectrometry (GC–MS)

A Trace GC 2000 Series gas chromatograph equipped with a Thermo Scientific DSQ II mass spectrometer was used. The GC–MS was first calibrated with pure reference compounds (representative of the major classes of compounds present in suberin) relative to *n*-hexadecane (internal standard). Compounds identification was based on the equipment spectral library (Wiley-Nist) and on previously published data, focussing their EI-MS fragmentation patterns and/or retention times.[30,31,38,39] Replicates were done to guarantee low variability and each analysis repeated twice. Each suberin sample was analysed by two complementary methods:

- Method 1, samples were converted to the corresponding trimethylsilyl (TMS) derivatives and analysed by GC–MS. In brief, suberin samples (*ca.* 15 mg) were reacted with 250 µL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide and 50 µL of trimethylchlorosilane in 250 µL of pyridine, during 30 min at 70 °C.[40]

- Method 2, samples were submitted to alkaline hydrolysis prior to the silylation, to release hydrolysable monomeric constituents. Briefly, suberin samples were treated

with a solution of 0.5 M NaOH in methanol/water (1:1, v/v) at 95 °C, during 4 hours.[41] The mixture was cooled to room temperature, acidified to pH 3-3.5 with 1 M HCl, and extracted three times by dichloromethane / water partition. The combined organic extracts were dried in a rotary evaporator, then trimethylsilylated as mentioned above, and analysed by GC–MS. The aqueous phases were also concentrated in a rotary evaporator and their content in glycerol analysed by HPLC.

3.7 High Performance Liquid Chromatography

Samples were analysed by HPLC, using a Waters chromatographer, consisting of a 510 Pump, a 715 Autosampler and a Temperature Control Module (Waters Chromatography, Milford, MA, USA), connected to a LKB 2142 Differential Refractometer detector (Bromma, Sweden). Data acquisition was accomplished with the Millenium32 v3.05.01, 1998 system (Waters). Chromatographic separation was undertaken at 60 °C using an Aminex HPX-87H column (300 x 7.8 mm) with 9 μm particle size (Bio-Rad, Hercules, California). Elution was carried out isocratically, at a flow rate of 0.5 $mL \cdot min^{-1}$, with 0.005 N of H_2SO_4 and the sample volume injected was 20 μL. Lactic acid (retention time = 15.98 min) and glycerol (retention time = 15.20 min) quantifications were done using an external calibration curve with high purity standards and within the quantification limits of 0.25 - 20.41 $mg \cdot mL^{-1}$ and 0.25 - 9.99 $mg \cdot mL^{-1}$, respectively.

3.8 Reaction of standard compounds in cholinium hexanoate media

Tests of cholinium hexanoate ability to cleave standard compounds were done in the same conditions used for cork, *i.e.* 100 °C, with stirring (experimental triplicates and technical duplicates).

Glyceryl trioctanoate and octyl octanoate were mixed with cholinium hexanoate at a 1.5 ratio of ionic liquid moles *per* mole of ester bonds, during 1, 2, 4 or 8 hours. At the end of the test, after partition of the ensuing mixture in dichloromethane / water, the organic layer was recovered, concentrated in a rotary evaporator, dried at 50 °C, and then analysed by GC-MS as described in GC-MS - Method 1. The aqueous phases were also concentrated in a rotary evaporator and their content in glycerol analysed by HPLC.

The remaining standard compounds, namely 9,10-epoxy-18-hydroxyoctadecanoic acid, poly(12-hydroxydodecanoic acid) and poly(lactic acid) were mixed with cholinium hexanoate for 4 hours, using a mass ratio (standard: cholinium hexanoate) of 1:4, 1:3, 1:10, 1:10 and 1:10, respectively. For the first two compounds, at the end of the test, an excess of water was added, the precipitate recovered by centrifugation, dried and analysed by ATR-FTIR and/or NMR. In the test with poly(lactic acid), after the reaction, an excess of water was added, the insoluble residue recovered by filtration, dried and then analysed by ATR-FTIR. The content of lactic acid in the aqueous phase was determined by HPLC.

3.9 Thermal microscopy

Thermal microscopy analyses were done using a Leitz Orthoplan polarizing microscope (Wetzlar, Germany) equipped with a JVC digital camera (TK-C130), a Linkham hot stage with a TMS90 temperature controller (±0.5 °C) and a CS196 cooling system.

3.10 Computational Methods

Density functional theory (DFT)[42,43] calculations were performed with the Gaussian 09 software package[44] with the hybrid functional PBE1PBE,[45-48] and the 6-31+G** basis set. Full geometry optimisations including solvent effects (DMSO) were carried out, using the polarisable continuum model (PCM)[49]. The starting structures are based in the most stable approach geometry of the ionic liquid and reactants. Harmonic vibrational frequencies were calculated for all located stationary structures to verify whether they were minima or transition states. Zero-point energies and thermal corrections were taken from unscaled vibrational frequencies. Free energies of activation are given at 25 ºC. All energies were calculated relative to the reagents. All bond lengths are in angstrom (Å) and energies in $kJ \cdot mol^{-1}$.

4. Results and Discussion

The cholinium-hexanoate-based method for suberin extraction combines partial depolymerisation of suberin with high selectivity and extraction efficiency.[32-34] To

identify the chemical bonds prone to be cleaved in the cholinium hexanoate media, we carried out a comprehensive study of the lability of suberin and of standard compounds carrying the key structural bonds in suberin, namely linear aliphatic esters and acylglycerol esters, as depicted schematically in Figure 1.

4.1 Suberin depolymerisation occurs through cleavage of ester bonds: time-course analysis

The natural abundance of suberin in cork is typically *ca.* 50-60 wt%.[10] The extraction of suberin from cork with cholinium hexanoate was extremely fast, given that after one hour the recovery yield was 46.8 wt%. However, after this phase the suberin recovery yield showed a minor increase over time, reaching 54.7 wt% at the eighth hour of extraction (Table 1). Thermal microscopy analysis showed a rapid disruption of cork during the first hour of the extraction. No significant differences were observed for longer periods. Suberin extraction was probably facilitated by this disruption, which exposes the cell wall components to the ionic liquid phase (Supplementary Section S1).

Aiming to identify which chemical bonds in suberin were cleaved in the cholinium hexanoate media, structural composition analyses of the samples recovered

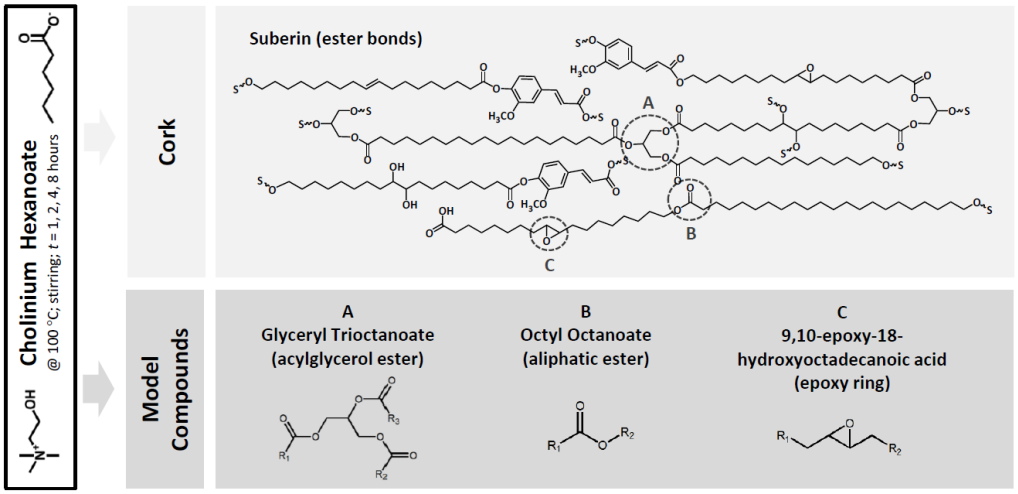


Figure 1| Schematic view of the experiments undertaken to identify the key ester bonds and epoxy groups in suberin prone to be cleaved in the cholinium hexanoate media. Cholinium hexanoate tests with cork (top) and with standard compounds (bottom). The standard compounds reproduce main chemical functionalities present in suberin, namely acylglycerol ester (A), aliphatic ester (B), epoxy ring (C).

along the extraction time were undertaken. After eight hours of extraction, the ester features, namely the resonance at δ 173 ppm (^{13}C CP/MAS NMR, Figure 2A) and the carbonyl stretching at 1731 cm^{-1} (ATR-FTIR, Figure 2B), were still detected in the corresponding spectra. However, the intensity of the ester resonance at δ 173 ppm decreased along the extraction time, suggesting ester bond cleavage. In addition, the peak at 1715 cm^{-1}, which was barely distinguishable in the first hours of the extraction, for longer periods became a defined peak. This peak is associated with hydrogen bonded carbonyl groups in either esters or acids (see magnification in Figure 2B), corroborating the idea that ester bond cleavage occurred. It becomes apparent that the ionic liquid led to mild depolymerisation of suberin through continuous ester bond cleavage.

Table 1| Analysis of suberin extracted from cork by cholinium hexanoate. Values depicted correspond to suberin recovery yield [100•(m recovered suberin / m cork], solubility in dichloromethane [100•(m soluble suberin / m suberin] and alkaline hydrolysis recalcitrancy [100-(100•(m hydrolysable suberin / m suberin] and to the quantification of the glycerol released during suberin depolymerisation, namely in the cholinium hexanoate media (Glycerol[ChHex]) and by alkaline hydrolysis (Glycerol[NaOH]). The second quantifies the hydrolysable glycerol that remained in suberin after the depolymerisation of suberin in the ionic liquid media. Values in brackets stand for standard deviation between replicates.

time / h			**1**	**2**	**4**	**8**
Recovery Yield (STD) / wt%			46.8 (4.5)	47.3 (0.9)	51.0 (5.8)	54.7 (14.8)
Solubility in CH_2Cl_2 (STD) / wt%			46.2 (0.3)	48.0 (1.1)	55.7 (2.9)	65.2 (1.7)
Recalcitrancy to Alkaline Hydrolysis Yield (STD) / wt %			34.4 (3.9)	37.7 (12.4)	49.0 (7.8)	46.3 (0.1)
Glycerol $mg_{glycerol} / g_{suberin}$	**Glycerol$_{[ChHex]}$** [†]	HPLC	17.4 (3.0)	18.6 (3.1)	51.1 (4.1)	63.0 (21.2)
		GC-MS	4.3 (0.4)	5.5 (2.3)	2.8 (1.0)	2.7 (2.5)
		Total	*21.7*	*24.1*	*53.9*	*65.7*
	Glycerol$_{[NaOH]}$ [‡]	HPLC	34.9 (4.5)	32.2 (3.2)	7.6 (1.9)	0.0 (0.0)
		GC-MS	0.8 (0.3)	0.7 (0.2)	0.4 (0.3)	0.3 (0.1)
		Total	*35.7*	*32. 9*	*8.0*	*0.3*
	Total Glycerol		*57.4*	*57.0*	*61.9*	*66.0*

[a] Glycerol$_{[ChHex]}$, glycerol released during suberin depolymerisation in the cholinium hexanoate media, calculated as the total of the glycerol detected by HPLC (*i.e.* solubilised in water during the filtration and the precipitation steps) and by GC-MS (*i.e.* present in the sample).

[b] Glycerol$_{[NaOH]}$, glycerol released during alkaline hydrolysis of suberin samples, calculated as the total of the glycerol detected by HPLC (*i.e.* solubilised in the aqueous phase during dichloromethane / water partition after the alkaline hydrolysis) and by GC-MS (in the sample after alkaline hydrolysis)

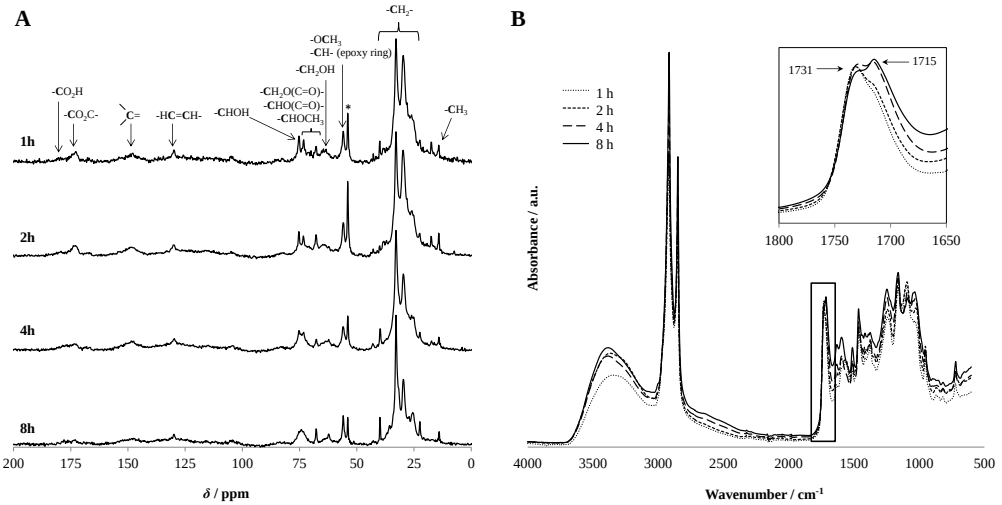


Figure 2| ^{13}C CP/MAS NMR spectra (A) and ATR-FTIR spectra (B) of suberin extracted with cholinium hexanoate after 1, 2, 4 or 8 hours.

Other important observations in the ^{13}C CP/MAS NMR spectra of the recovered suberin samples are the two major resonances at δ 33 and 30 ppm which are associated with aliphatic methylenes (Figure 2A). The ratio 33/30 ppm increased over the extraction time. The carbons at δ 33 ppm have been suggested to report slightly lower mobility in comparison to those at δ 30 ppm[31]. This has been related to the presence of methylene groups near oxygen vicinities (suggestive of linkage to lignin and polysaccharides),[31] or to the presence of crystalline/recalcitrant domains[50,51]. Other resonances typical of suberin can be noticed in all spectra, namely signals at δ 50 - 90 ppm, δ 130 and 148 ppm, assigned to carbons linked to oxygen, vinylic and quaternary carbons, respectively. One cannot disregard the hypothesis that cholinium hexanoate contamination contributed to the resonance at δ 54 ppm (more intense in the samples extracted for one or two hours, see * in Fig. 2A).

The ATR-FTIR spectra of the suberin samples show two major peaks at 2918, 2851 cm^{-1} and a band between 3679-3034 cm^{-1} (Figure 2B). These are respectively associated to the aliphatic chains and to the hydroxyl groups in carboxylic acids and/or alcohols. The hydroxyl band increased along the extraction time. This can be related to the release of hydroxyl moieties, which reinforce the idea that suberin depolymerisation was continuous along the course of the extraction.

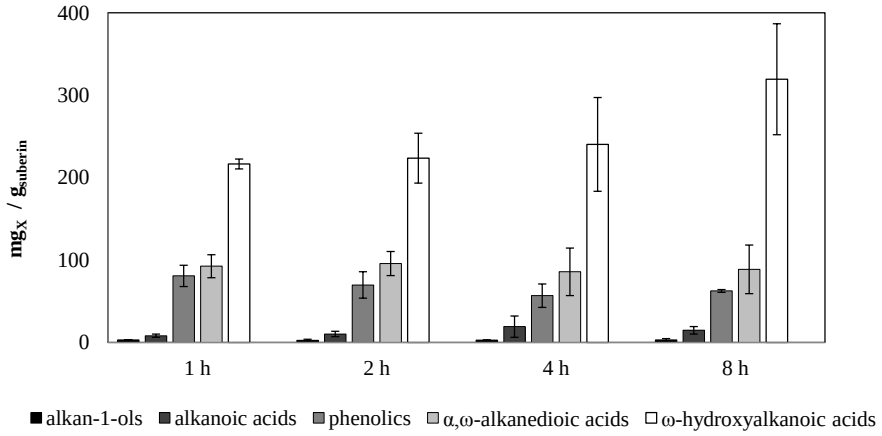


Figure 3| Quantification of suberin monomers in the samples recovered after depolymerisation in cholinium hexanoate media and submitted to alkaline hydrolysis prior to the GC-MS analysis (GC-MS – Method 2). Detailed monomeric quantification in Supplementary Section S2.

More information on the monomeric composition of suberin along the extraction time can be retrieved using GC-MS analysis. The underlying concept here is that suberin extracted with cholinium hexanoate is still cross-linked and/or oligomeric. Consequently, the high molecular weight of these structures hampers their detection by GC-MS (see GC-MS - Method 1, Experimental section). In agreement, very low identification yields were attained (*ca.* 2 - 5 wt%), notwithstanding that the main typical suberin monomers could be detected (data not shown). Monoacylglycerol dimers were only detected in the suberin samples recovered after the first two hours of the extraction, reinforcing the idea that, until the end of the extraction time, oligomers released to the ionic liquid media were further depolymerised.

Aiming to overcome the low GC-MS identification yields, any hydrolysable chemical bonds in the ionic liquid extracted suberin were cleaved by conventional alkaline hydrolysis before the analysis (see GC-MS - Method 2, Experimental section). Consequently, the identification yields of these samples were significantly higher, *ca.* 50 wt% (Supplementary Section S2). In general, the amounts of the monomers herein identified were coherent with those typically reported for suberin extracted with conventional methods.[17,30,31] Major compounds identified were ω-hydroxyalkanoic acids, followed by α,ω-alkanedioic acids and phenolics, and minor ones were alkanoic acids and

alkan-1-ols (Figure 3, Supplementary Section S2). Similar to previous reports,[17] the most abundant monomers herein detected were 22-hydroxydocosanoic acid, followed by ferulic acid, 9,10,18-trihydroxyoctadecanoic acid and 9,10-dihydroxyoctadecanedioic acid.

Glycerol is a major component of suberin,[10,17,52,53] however its high water solubility justifies that the quantities detected by GC-MS, either before or after alkaline hydrolysis of suberin, were very low (Table 1). Most glycerol released from suberin, due to extraction with cholinium hexanoate, becomes solubilised in water during the filtration and the precipitation steps. After alkaline hydrolysis the hydrolysed suberin fraction is recovered by dichloromethane / water partition, consequently most of the hydrolysed glycerol remains in the aqueous phase. In fact, the total glycerol obtained herein is comparable to that reported for conventionally extracted suberin (*ca.* 6 wt%, Table 1).[52,53]

Suberin depolymerisation with cholinium hexanoate released increasing quantities of glycerol, from 21.47 to 67.51 $mg_{glycerol}$ / $g_{suberin}$ ($glycerol_{[ChHex]}$, Table 1). On the contrary, the glycerol released from suberin during alkaline hydrolysis progressively decreases to zero along the extraction time ($glycerol_{[NaOH]}$, Table 1). This means that after eight hours, virtually all hydrolysable acylglycerol ester bonds present in suberin were cleaved in the ionic liquid media. However, significant amounts of suberin monomers were still released after alkaline hydrolysis. Both ester bonds present in suberin, *viz.* linear aliphatic and acylglycerol, are labile to the alkaline hydrolysis. Thus, it seems that the ionic liquid is efficient towards acylglycerol ester bonds, but fails to cleave most of the linear aliphatic esters bonds.

Suberin solubility in dichloromethane and its recalcitrancy to alkaline hydrolysis increased over the extraction time, 46.2 to 65.2 %wt and 34.4 to 46.3 %wt, respectively (Table 1). Upon rapid removal from the cell wall to the ionic liquid media, suberin oligomeric structures undergo continuous depolymerisation - hence solubility in dichloromethane increases. At the same time, non-hydrolysable suberin oligomeric structures are progressively removed from the cell wall - hence recalcitrance increases. All aforementioned data was integrated to propose a model for suberin extraction from cork in cholinium hexanoate media (Figure 4).

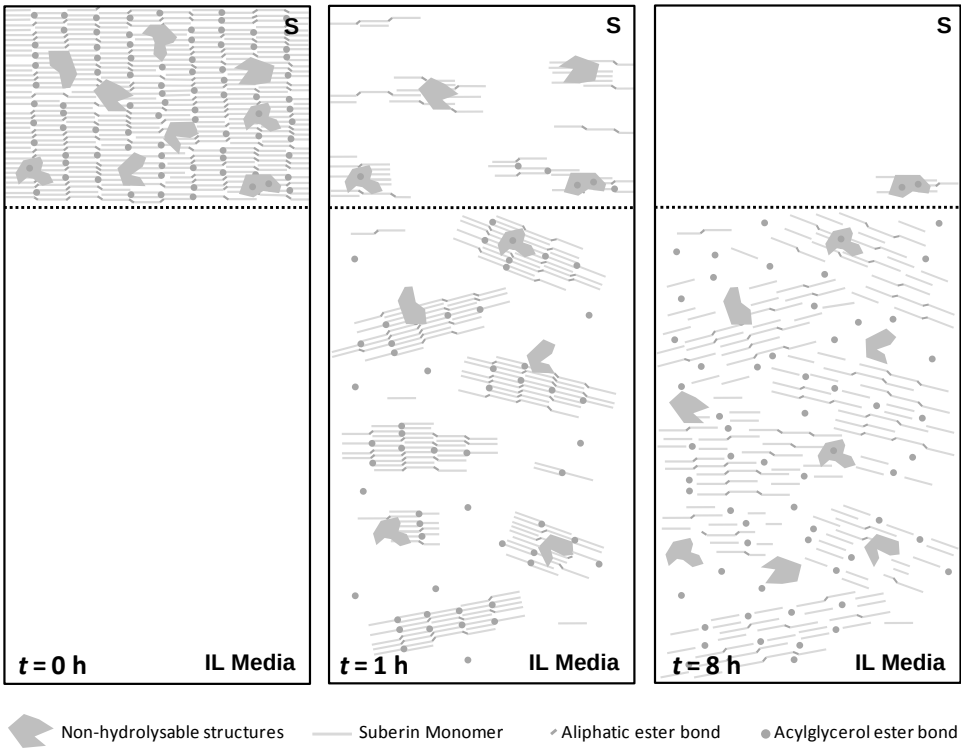


Figure 4| Schematic view of how cholinium hexanoate leads to suberin depolymerisation along the extraction time. S, suberin deposited in the cell wall; IL Media, Ionic liquid media.

4.2 Preferential cleavage of acylglycerol esters in standard compounds

Apparently cholinium hexanoate promoted efficient cleavage of the acylglycerol ester bonds in suberin, but not of the linear aliphatic ester bonds. To further support these findings, the ionic liquid ability to cleave standard compounds carrying linear aliphatic or acylglycerol ester bonds was analysed by monitoring along time the reaction of glyceryl trioctanoate or octyl octanoate, respectively (Figure 1). These standard compounds showed different reaction kinetics (Figure 5). Octyl octanoate was fairly resistant to cleavage. After eight hours of reaction only *ca.* 30 %wt was hydrolysed to octanoic acid and octanol. On the contrary, after the same reaction time, more than 80 %wt of the glyceryl trioctanoate was cleaved, releasing glycerol, octanoic acid and the corresponding diacyl- and monoacyl- glycerols (Figure 5). Based on the low amounts of 2-monoacylglycerol released, it is likely that the most favourable cleavage occurred in the

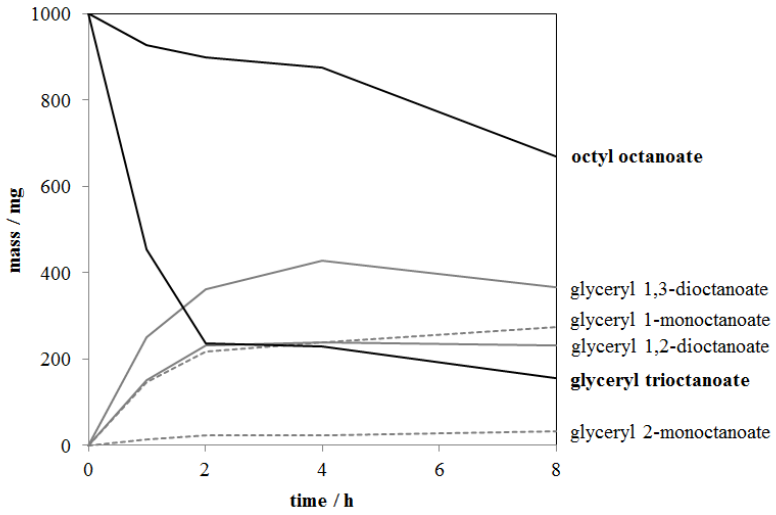


Figure 5| Compounds detected after treatment of glyceryl trioctanoate and octyl octanoate with cholinium hexanoate during 1, 2, 4 and 8 hours. All compounds were identified and quantified by GC-MS. Glycerol, octanol, octanoic acid and error bars were not represented for ease of visualisation (average standard error = 27.8 mg). At time zero, glyceryl trioctanoate and octyl octanoate were assumed to represent the only compounds present in the mixture.

acyl group at position C2 of glycerol. This is suggestive of possible steric hindrance and/or chemical selectivity in the reaction promoted by the ionic liquid. This partially explains why amongst the few monomers identified by GC-MS in the suberin samples extracted with cholinium hexanoate none was a 2-monoacylglycerol.[32,34]

The lack of efficiency of cholinium hexanoate to promote cleavage of linear aliphatic esters was further confirmed using model aliphatic polyesters. After four hours, no alterations were detected in the ATR-FTIR spectra of poly(lactic acid), in particular in the peaks assigned to C=O stretching. Moreover, after this reaction time the mass of the poly(lactic acid) recovered was *ca.* 91.61 ± 0.26wt%, agreeing with the amount of lactic acid detected (6.03 ± 0.63 wt%). The ATR-FTIR spectra of poly(12-hydroxydodecanoic acid) was also unaltered after four hours in the ionic liquid media.

Since the epoxy ring is a significant non-structural functionality in suberin its lability in the ionic liquid media was also preliminary analysed using 9,10-epoxy-18-hydroxyoctadecanoic acid. NMR analysis showed that while the epoxy ring was partially

preserved after 4 hours, some secondary alcohol signals were also detected (^{1}H: 3.5 ppm; ^{13}C: 72 ppm, data not shown). This observation, which was also confirmed by GC-MS and ESI-MS (data not shown), can be associated with the opening of the epoxy ring into a *vic*-diol and/or methoxyhydrin, as suggested above by the ^{13}C CP/MAS NMR data of suberin samples.

Suberin is a structural component of the cork cell wall, thus cannot be removed without impairing its integrity (Supplementary Section S1). The other major polymers in the cork cell wall are likely to be also exposed to the ionic liquid media, namely polysaccharides and lignin, which are linked *via* ether bonds and ether/C–C bonds, respectively. Preliminary data obtained with α-cellulose and alkali lignin, suggested that cholinium hexanoate was unable to promote hydrolysis of their inter-monomeric bonds (data not shown). This further supports cholinium hexanoate selectivity towards suberin.[32-34]

4.3 Cholinium hexanoate as catalyst in the hydrolysis of ester bonds

The cleavage of ester bonds promoted by cholinium hexanoate led to the release of free alcohols and carboxylic acids from glyceryl trioctanoate and octyl octanoate (Figure 5), as well as from suberin (GC-MS Method 1, data not shown). This brings further light to this process: the ester bonds were hydrolysed, implying that the ionic liquid plays the dual role of solvent and catalyst. Water was present in the reaction media, since it was *ca.* 0.2 %wt in cholinium hexanoate and *ca*. 2.4 wt% in cork[34]. Thus the molar fraction of water was 4.9 % relative to the ionic liquid (0.42 wt% in the reaction media and 8.4 wt% relative to suberin).

While the capacity of ionic liquids to play the dual role of solvent and catalyst has been well described,[54] the complex set of chemical and physical interactions, including Coulombic and dipole forces, hydrogen-bonding and acid-base interactions, behind the augmented reactivity in an ionic liquid media are not yet fully understood. Up to now, few hydrolysis reactions in ionic liquid media have been reported, associated to the nucleophilic activation of water.[54,55] It has been demonstrated that, when present at low concentrations, water is dispersed in the ionic liquid as single molecules.[54,55] They form

specific electrostatic interactions and hydrogen-bonding with the ions. As the concentration of water increases, self-association of the water molecules and ion clustering probably occurs, weakening the catalytic effect of the ionic liquid. The chemistry of water in these domains is still a puzzling scientific question.

It was demonstrated that the hydrolysis kinetics is influenced by the ionic liquid hydrogen bonding donor and acceptor ability.[56] Cholinium carboxylates are good hydrogen bond acceptors (Kamlet Taft parameters $\beta > 0.9$)[57]. Accordingly, the mechanism of suberin hydrolysis in cholinium hexanoate media might be related to the nucleophilic activation of water. In fact, increasing the molar fraction of water in the reaction media from 4.9 % to 40%, led to a dramatic reduction in the extraction efficiency of cholinium hexanoate (from 51.0 ± 5.8 to 25.3 ± 2.4 wt%). Water in excess hampered the ionic liquid catalytic effect and/or acted as an anti-solvent. Hence, during suberin depolymerisation from cork, a fine balance of the water concentration in the reaction media needs to be attained.

4.4 Cholinium hexanoate catalyses ester hydrolysis: computational study

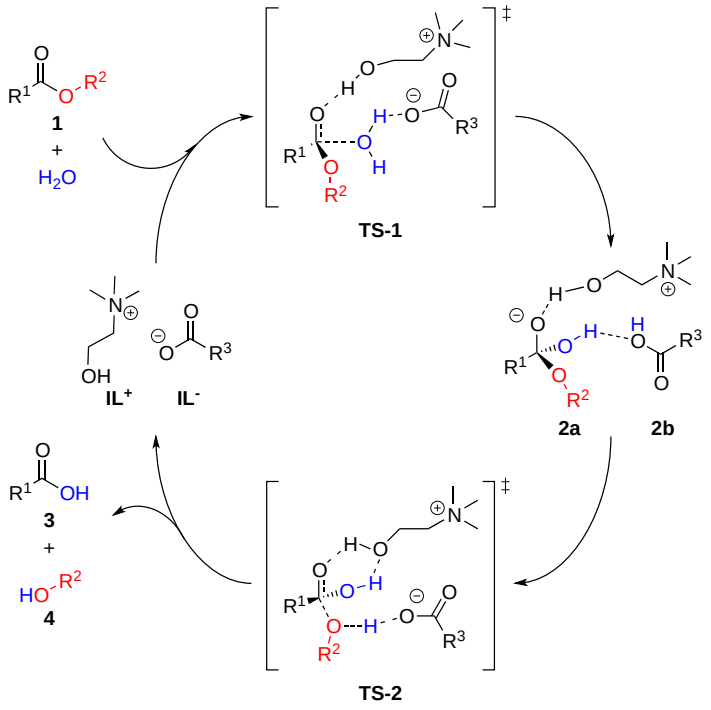
According to the experimental results, cholinium hexanoate catalysed the hydrolysis of esters originating acids and alcohols. A density functional theory (DFT) study[42,43] was performed in order to propose a reaction mechanism. All the intermediates and transitions states (TSs) along the possible reaction pathways were calculated. The proposed reaction mechanism consists of two steps (Scheme 1). In the first step, the hydroxyl group of the cholinium cation (IL^+) establishes one hydrogen bond with the oxygen of the carbonyl group of the ester, favouring the nucleophilic attack of water at the carbon (TS-1). The intermediate **2a** has the newly formed OH bond. In the second step, one proton is transferred from **2b** to the $O(R^2)$ atom, weakening the C-O bond (TS-2). The products, the acid **3** and the alcohol **4**, are formed and the ionic liquid regenerated, closing the catalytic cycle.

The activation barriers obtained for the hydrolysis of four types of linear aliphatic esters (methyl and ethyl acetate, butyl butyrate, and octyl octanoate) catalysed by cholinium acetate or hexanoate are depicted in Table 2 and Figure 6, and show that the

second step of the reaction is the limiting one. The energy difference between TS-1 and TS-2 (Δ (TS-2 – TS-1)) increases with the size of the ester carbon chain, as the linear ester model becomes closer to reality. This arises essentially from the destabilisation of TS-2, since the close proximity of the carbon chain R^1 and R^2 increases the steric repulsion and thus the activation energy. The calculated activation energies are higher in the reaction catalysed by cholinium hexanoate than by cholinium acetate, reflecting the slightly higher pK_a of hexanoic acid (4.88 *vs* 4.76 of acetic acid). This difference is, however, too small and not significant considering the nature of the model, as the substrates are linear aliphatic esters and the trends may easily be reversed. Since cholinium hexanoate efficiency towards the extraction of suberin was 1.5 fold higher than that of cholinium acetate,[33] this is most probably due to its superior solvent ability towards aliphatic chains. This further emphasises the dual role of the cholinium hexanoate, both as solvent and as catalyst.

The experimental results also showed that the hydrolysis catalysed by cholinium hexanoate was faster in triacylglycerol esters than in monoacylglycerol esters (Figure 5), and that triacylglycerol esters were preferential hydrolysed at the C2 position (Table 3). The reaction mechanism was studied considering the hydrolysis of glyceryl triacetate, catalysed by cholinium acetate, at both positions C1 and C2 (Table 3). The second step (TS-2) is again the limiting one for the reaction at both carbon centres. In addition, the reaction at C2 is faster than at C1 (lower activation energies), in agreement with the experimental results (Figure 5). Thus, as suggested above, it seems that both steric hindrance and chemical reactivity play a role in the hydrolysis of acylglycerol esters in the ionic liquid media.

Finally, the activation energies for the hydrolysis of glyceryl triacetate catalysed by cholinium acetate (Table 3) are lower than those of the linear aliphatic esters (Table 2). This can be correlated to the higher electrophilicity of the ester oxygen atom in the triacylglycerol than in the linear aliphatic ester. These data suggest how cholinium hexanoate might promote a mild depolymerisation of suberin from cork.



Scheme 1| Proposed mechanism for the hydrolysis of esters catalysed by cholinium alkanoates. TS and IL stand for transition state and ionic liquid, respectively.

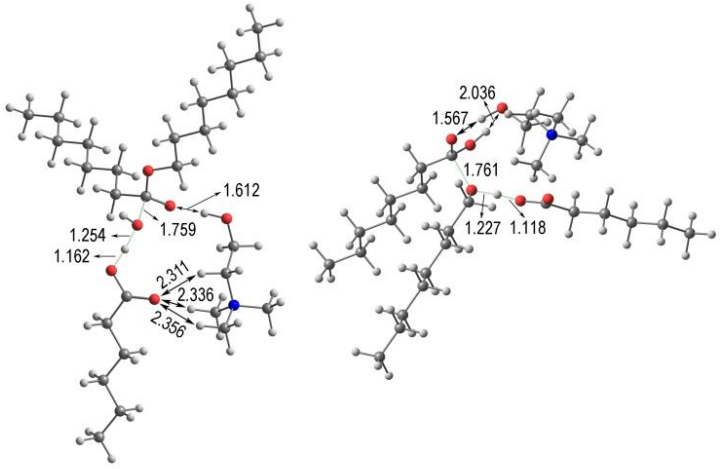
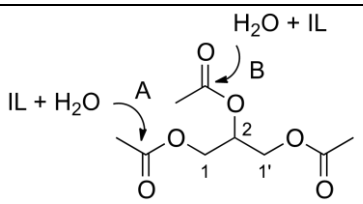


Figure 6| Calculated transition states (TSs) structures for the two steps of the hydrolysis of octyl octanoate catalysed by cholinium hexanoate. All bond lengths are in angstrom (Å).

Table 2| Gibbs activation energies (kJ.mol^{-1}) calculated for the transition states (TSs) presented in Scheme 1.

Ionic liquid	Transition state	Ester			
		methyl acetate	ethyl acetate	butyl butyrate	octyl octanoate
Cholinium acetate	**TS-1**	151.6	153.7	154.8	154.1
	TS-2	151.9	157.4	160.5	163.2
	Δ (**TS-2** – **TS-1**)	0.3	3.7	5.7	9.1
Cholinium hexanoate	**TS-1**	153.6	154.3	156.4	155.5
	TS-2	155.0	161.5	164.2	166.6
	Δ (**TS-2** – **TS-1**)	1.4	7.2	7.8	11.1

Table 3| Gibbs activation energies (kJ.mol^{-1}) calculated for the hydrolysis of glyceryl triacetate catalysed by cholinium acetate.

Transition State		
	Attack A (C1)	**Attack B (C2)**
TS-1	147.8	144.3
TS-2	151.8	146.5
Δ (**TS-2** – **TS-1**)	4.0	2.2

5. Conclusions

Two distinct phases describe the extraction of suberin from cork with cholinium hexanoate. First, rapid removal of nearly whole suberin, still highly polymerised and cross-linked, occurs. This is followed by slow removal of recalcitrant structures, non-hydrolysable and displaying a high cross-link density, concomitantly with continuous depolymerisation of labile oligomers dispersed in the ionic liquid media (Figure 4). The ionic liquid plays the dual role of solvent and catalyst and leads to suberin efficient

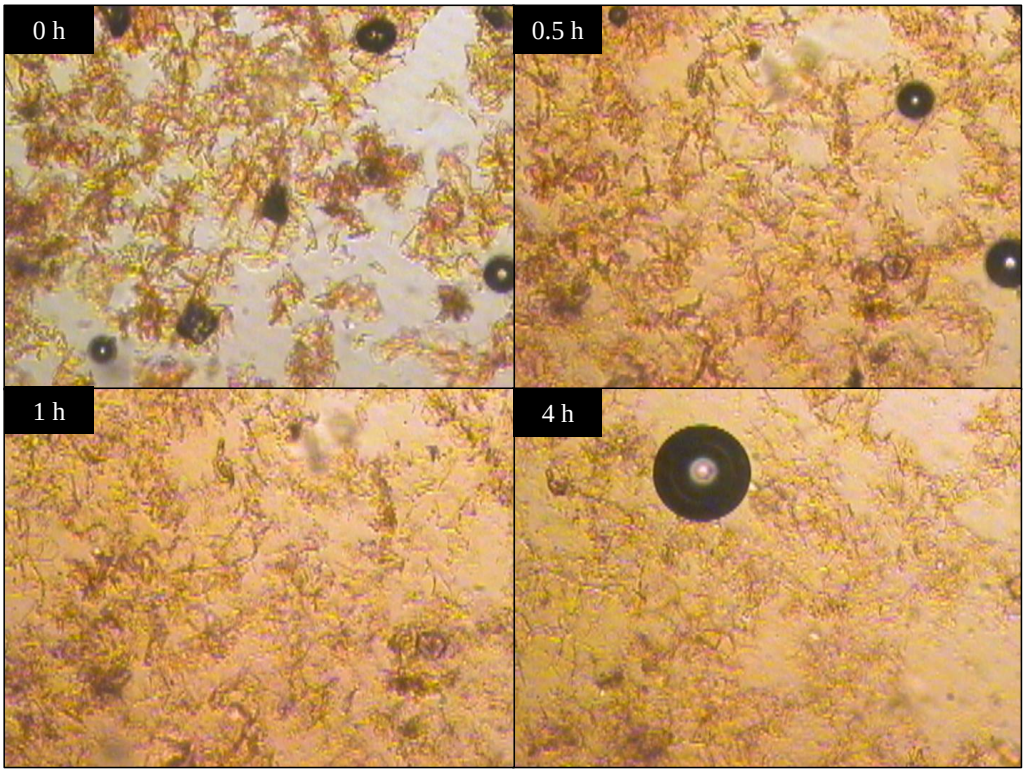
extraction through a mild depolymerisation. Disruption of the three-dimensional network of suberin in the ionic liquid media, progresses by preferential cleavage of acylglycerol ester bonds (with the faster kinetics in C2), whilst the linear aliphatic ester bonds remain largely preserved. This reaction mechanism leads to the efficient recovery of a high cross-linked and thus esterified suberin, thus different from that extracted with conventional methods which promote thorough ester cleavage. The ionic liquid catalyses the hydrolysis of esters, hence the water concentration in the reaction media is a decisive parameter. At high concentrations, water weakens the catalytic effect of cholinium hexanoate and/or acts as anti-solvent. A DFT study of the reaction mechanism showed that cholinium hexanoate catalyses the nucleophilic attack of water in two steps. This work should inspire the isolation of biopolyesters from numerous renewable resources, aiming at the development of strategies for their valorisation. A new chemical reaction for the hydrolysis of lipids catalysed by ionic liquids is now available. Ionic liquid chemistry opens the possibility to achieve designer solvents of high performance displaying high selectivity towards a specific carbon centre in an ester bond.

6. Acknowledgements

R. F., A. F. S. and F. J. S. D. are grateful to Fundação para a Ciência e a Tecnologia (FCT), Portugal, for the fellowships SFRH/BD/48286/2008, SFRH/BPD/73383/2010 and SFRH/BPD/76878/2011, respectively; H. G. is indebted to Fundação Calouste Gulbenkian, Portugal, for the fellowship 21-95587-B. The work was partially supported by a grant from Iceland, Liechtenstein and Norway through the EEA financial mechanism (Project PT015), by FCT through the Grants PEst-OE/EQB/LA0004/2011, Pest-C/CTM/LA0011/2011, PEst-OE/QUI/UI0612/2013, PTDC/QUI-QUI/120982/2010 and REDE/1517/RMN/2005, and by a cooperation grant from FCT and the Deutscher Akademischer Austauschdienst (DAAD). The authors wish to thank Maria Cristina Leitão for the acquisition of HPLC analyses.

7. Supplementary Information

Section 1 (S1)| Polarised light thermal microscopy images of cork during suberin extraction with cholinium hexanoate at discrete times (amplification 20×).



Section 2 (S2)| Main monomers identified by GC–MS analysis of suberin samples after alkaline hydrolysis. Results are given in mg of compound per gram of dried starting material.

Compound, x	$m_x/m_{suberin}$ mg/g (Method 2)			
	1 hours	2 hours	4 hours	8 hours
Alkan-1-ols	***3.26***	***2.60***	***2.88***	***3.32***
tetracosanol	3.26	2.60	2.88	3.32
Alkanoic acids	***8.22***	***10.28***	***19.37***	***14.84***
hexanoic acid[a)]	0.55	0.51	0.16	0.62
hexadecanoic acid	1.07	1.81	8.32	3.34
octadecanoic acid	0.79	1.22	2.25	1.31
octadec-9-enoic acid	0.53	0.59	1.47	0.77
docosanoic acid	4.15	4.00	4.78	7.03
tetracosanoic acid	1.68	2.66	2.55	2.39
ω-Hydroxyalkanoic acids	***216.61***	***223.65***	***240.39***	***319.50***
16-hydroxyhexadecanoic acid	1.91	1.62	1.25	1.86
9,10,18-trihydroxyoctadecanoic acid	56.29	65.41	89.14	101.06
11,12,18-trihydroxyoctadecenoic acid	1.33	1.67	1.19	1.25
18-hydroxyoctadec-9-enoic acid	28.18	28.18	20.53	33.00
9,18-dihydroxy,10-methoxyoctadecanoic acid[b)]	15.60	8.74	0.84	0.00
20-hydroxyeicosanoic acid	5.96	5.72	6.37	6.96
11,12,20-trihydroxyeicosanoic acid	1.75	2.95	4.46	4.18
22-hydroxydocosanoic acid	88.82	89.46	96.67	142.37
24-hydroxytetracosanoic acid	16.77	19.90	19.95	28.83
α,ω-Alkanedioic acids	***92.66***	***95.80***	***85.87***	***88.82***
hexadecanedioic acid	3.42	3.02	1.85	2.08
octadecanedioic acid	1.06	1.08	1.00	0.84
9,10-dihydroxyoctadecanedioic acid	50.29	51.38	49.82	44.32
9-hydroxy,10-methoxyoctadecanedioic acid [b)]	2.41	1.87	0.42	0.63
octadec-9-enedioic acid	8.40	6.99	3.92	4.96
9,10-dihydroxyeixosanedioic acid	1.56	3.23	3.35	2.33
eicosanedioic acid	2.98	2.94	1.56	2.76
docosanedioic acid	18.47	20.43	18.85	27.55
tetracosanedioic acid	4.05	4.86	5.08	3.36
Phenolics	***80.88***	***69.81***	***56.94***	***62.59***
4-hydroxy-3-methoxybenzoic acid (vanillic acid)	7.62	4.35	2.95	3.08
4-hydroxy-3-methoxy-cinnamic acid (*trans*-ferulic acid)	15.71	16.80	11.14	9.89
4-hydroxy-3-methoxy-cinnamic acid (*cis*-ferulic acid)	57.54	48.67	42.85	49.62
Extractives	***23.67***	***36.94***	***24.19***	***19.85***
β-sitosterol	1.40	4.65	9.31	1.45

(continued)

Compound, x	$m_x/m_{suberin}$ mg/g (Method 2)			
	1 hours	**2 hours**	**4 hours**	**8 hours**
friedelin	8.67	10.44	3.58	3.32
betulinol	10.29	14.44	5.93	11.65
betulinic acid	3.31	7.41	5.37	3.42
Glycerol	***0.82***	***0.74***	***0.40***	***0.31***
Unidentified long chain fatty acid derivatives	***64.64***	***53.86***	***5.45***	***14.17***
Identification Yield (wt%)	49.07	49.37	43.55	52.34

a) Hexanoic acid corresponds to the ionic liquid anion, thus it was not accounted for the identification yield.
b) Methoxyhydrin artefacts from the corresponding epoxyacid.

Section 3 (S3)| Cartesian coordinates and computed total energies.

The Cartesian coordinates and computed total energies for the following systems, have been calculated: 1) reactants, 2) reaction between cholinium acetate and methyl acetate, 3) reaction between cholinium hexanoate and methyl acetate, 4) reaction between cholinium acetate and ethyl acetate, 5) reaction between cholinium hexanoate and ethyl acetate, 6) reaction between cholinium acetate and butyl butyrate, 7) reaction between cholinium hexanoate and butyl butyrate, 8) reaction between cholinium acetate and octyl octanoate, 9) reaction between cholinium hexanoate and octyl octanoate and 10) reaction between cholinium acetate and glyceryl triacetate. These data is extremely extensive and are not within the scope of this thesis, thought it can be provided upon request.

8. References

1. A. Gandini, The irruption of polymers from renewable resources on the scene of macromolecular science and technology, *Green Chem.*, 2011, **13**, 1061-1083.
2. P. E. Kolattukudy, Structure, biosynthesis, and biodegradation of cutin and suberin, *Annu. Rev. Plant Physiol.*, 1981, **32**, 539-567.
3. A. Heredia, Biophysical and biochemical characteristics of cutin, a plant barrier biopolymer, *Biochim. Biophys. Acta*, 2003, **1620**, 1-7.
4. K. Ranathunge, L. Schreiber and R. Franke, Suberin research in the genomics era—New interest for an old polymer, *Plant science*, 2011, **180**, 399-413.
5. L. Schreiber, Transport barriers made of cutin, suberin and associated waxes, *Trends Plant Sci.*, 2010, **15**, 546-553.
6. M. Pollard, F. Beisson, Y. Li and J. B. Ohlrogge, Building lipid barriers: Biosynthesis of cutin and suberin, *Trends Plant Sci.*, 2008, **13**, 236-246.
7. F. Beisson, Y. Li-Beisson and M. Pollard, Solving the puzzles of cutin and suberin polymer biosynthesis, *Curr. Opin. Plant Biol.*, 2012, **15**, 329-337.
8. M. A. Bernards, Demystifying suberin, *Can. J. Bot.*, 2002, **80**, 227-240.
9. A. Gandini, C. P. Neto and A. J. D. Silvestre, Suberin: A promising renewable resource for novel macromolecular materials, *Prog. Pol. Sci.*, 2006, **31**.
10. H. Pereira, Cork: biology, production and uses, *Elsevier*, Amsterdam, The Netherlands, 1st edn., 2007.
11. R. B. Franke, I. Dombrink and L. Schreiber, Suberin goes genomics: Use of a short living plant to investigate a long lasting polymer, *Front. Plant Sci.*, 2012, **3**, 1-8.
12. P. E. Kolattukudy and V. P. Agrawal, Structure and composition of aliphatic constituents of potato tuber skin (suberin), *Lipids*, 1974, **9**, 682-691.
13. R. G. Riley and P. E. Kolattukudy, Evidence for covalently attached *p*-coumaric acid and ferulic acid in cutins and suberins, *Plant Physiol.*, 1975, **56**, 650-654.
14. P. E. Kolattukudy, Biopolyester membranes of plants: Cutin and suberin, *Science*, 1980, **208**, 990-1000.
15. M. A. Bernards, in *Encyclopedia of Life Sciences (http://www.els.net)*, John Wiley & Sons, Ltd, Chichester, 2002.
16. M. A. Bernards, Demystifying suberin, *Can. J. Bot.-Rev. Can. Bot.*, 2002, **80**, 227-240.
17. A. Gandini, C. Pascoal Neto and A. J. D. Silvestre, Suberin: A promising renewable resource for novel macromolecular materials, *Prog. Pol. Sci.*, 2006, **31**, 878-892.
18. J. Graça and S. Santos, Suberin: a biopolyester of plants' skin, *Macromol. Biosci.*, 2007, **7**, 128-135.
19. P. Sitte, Zum Feinbau der Suberinschichten im Flaschenkork, *Protoplasma*, 1962, **54**, 555-559.
20. R. E. Stark and J. R. Garbow, Nuclear magnetic resonance relaxation studies of plant polyester dynamics: 2. Suberized potato cell wall, *Macromolecules*, 1992, **25**, 149-154.
21. M. H. Lopes, A. Sarychev, C. Pascoal Neto and A. M. Gil, Spectral editing of ^{13}C CP/MAS NMR spectra of complex systems: Application to the structural characterisation of cork cell walls, *Solid State Nucl. Mag.*, 2000, **16**, 109-121.
22. M. A. Bernards and N. G. Lewis, The macromolecular aromatic domain in suberized tissue: A changing paradigm, *Phytochemistry*, 1998, **47**, 915-933.
23. M. A. Bernards, M. L. Lopez, J. Zajicek and N. G. Lewis, Hydroxycinnamic acid-derived polymers constitute the polyaromatic domain of suberin, *J. Biol. Chem.*, 1995, **270**, 7382-7386.

24. J. Negrel, B. Pollet and C. Lapierre, Ether-linked ferulic acid amides in natural and wound periderms of potato tuber, *Phytochemistry*, 1996, **43**, 1195-1199.
25. J. Graça, Hydroxycinnamates in suberin formation, *Phytochem. Rev.*, 2010, **9**, 85-91.
26. N. Cordeiro, M. N. Belgacem, A. Gandini and C. Pascoal Neto, Urethanes and polyurethanes from suberin 2: synthesis and characterization, *Ind. Crop. Prod.*, 1999, **10**, 1-10.
27. N. Cordeiro, A. Blayo, N. M. Belgacem, A. Gandini, C. Pascoal Neto and J.-F. LeNest, Cork suberin as an additive in offset lithographic printing inks, *Ind. Crop. Prod.*, 2000, **11**, 63-71.
28. A. F. Sousa, A. Gandini, A. J. D. Silvestre, C. Pascoal Neto, J. J. Cruz-Pinto, C. Eckerman and B. Holmbom, Novel suberin-based biopolyesters: From synthesis to properties, *J. Polym. Sci. Pol. Chem.*, 2011, **49**, 2281-2291.
29. A. F. Sousa, A. Gandini, A. J. D. Silvestre and C. Pascoal Neto, Synthesis and characterization of novel biopolyesters from suberin and model comonomers, *Chem. Sus. Chem.*, 2008, **1**, 1020-1025.
30. R. Ekman and C. Eckerman, Aliphatic carboxylic acids from suberin in birch outer bark by hydrolysis, methanolysis, and alkali fusion, *Pap. Ja Puu-Pap. Timber*, 1985, **67**, 255-273.
31. M. H. Lopes, A. M. Gil, A. J. D. Silvestre and C. Pascoal Neto, Composition of suberin extracted upon gradual alkaline methanolysis of *Quercus suber* L. cork, *J. Agric. Food Chem.*, 2000, **48**, 383-391.
32. R. Ferreira, H. Garcia, A. F. Sousa, M. Petkovic, P. Lamosa, C. S. R. Freire, A. J. D. Silvestre, L. P. N. Rebelo and C. Silva Pereira, Suberin isolation from cork using ionic liquids: Characterisation of ensuing products, *New J. Chem.*, 2012, **36**, 2014-2024.
33. H. Garcia, R. Ferreira, M. Petkovic, J. L. Ferguson, M. C. Leitão, H. Q. N. Gunaratne, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Dissolution of cork biopolymers in biocompatible ionic liquids, *Green Chem.*, 2010, **12**, 367-369.
34. R. Ferreira, H. Garcia, A. F. Sousa, C. S. R. Freire, A. J. D. Silvestre, L. P. N. Rebelo and C. Silva Pereira, Isolation of suberin from birch outer bark and cork using ionic liquids: A new source of macromonomers, *Ind. Crop. Prod.*, 2013, **44**, 520-527.
35. M. Petkovic, J. L. Ferguson, H. Q. N. Gunaratne, R. Ferreira, M. C. Leitão, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Novel biocompatible cholinium-based ionic liquids—toxicity and biodegradability, *Green Chem.*, 2010, **12**, 643-649.
36. A. M. Gil, M. Lopes, J. Rocha and C. Pascoal Neto, A ^{13}C solid state nuclear magnetic resonance spectroscopic study of cork cell wall structure: The effect of suberin removal, *Int. J. Biol. Macromol.*, 1997, **20**, 293-305.
37. A. Olsson, M. Lindström and T. Iversen, Lipase-catalyzed synthesis of an epoxy-functionalized polyester from the suberin monomer *cis*-9,10-epoxy-18-hydroxyoctadecanoic acid, *Biomacromolecules*, 2007, **8**, 757-760.
38. N. Cordeiro, M. N. Belgacem, A. J. D. Silvestre, C. Pascoal Neto and A. Gandini, Cork suberin as a new source of chemicals: 1. Isolation and chemical characterization of its composition, *Int. J. Biol. Macromol.*, 1998, **22**, 71-80.
39. P. C. R. O. Pinto, A. F. Sousa, A. J. D. Silvestre, C. Pascoal Neto, A. Gandini, C. Eckerman and B. Holmbom, *Quercus suber* and *Betula pendula* outer barks as renewable sources of oleochemicals: A comparative study, *Ind. Crop. Prod.*, 2009, **29**, 126-132.
40. R. Ekman, The suberin monomers and triterpenoids from the outer bark of *Betula verrucosa* Ehrh., *Holzforschung*, 1983, **37**, 205-211.
41. A. F. Sousa, P. C. R. O. Pinto, A. J. D. Silvestre and C. Pascoal Neto, Triterpenic and other lipophilic components from industrial cork byproducts, *J. Agric. Food Chem.*, 2006, **54**, 6888-6893.

42. W. Koch and M. C. Holthausen, A chemist's guide to density functional theory, *Wiley-Vch*, New York, USA, 2nd edn., 2001.

43. R. G. Parr and W. Yang, Density-functional theory of atoms and molecules, *Oxford University Press*, Oxford, UK, 1st edn., 1989.

44. M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. Montgomery, J. A., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, O. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski and D. J. Fox, in *Gaussian Inc.*, Wallingford, CT, 2009.

45. J. P. Perdew, K. Burke and M. Ernzerhof, Generalized Gradient Approximation Made Simple, *Phys. Rev. Lett.*, 1996, **77**, 3865-3868.

46. J. P. Perdew, K. Burke and M. Ernzerhof, Generalized gradient approximation made simple, *Phys. Rev. Lett.*, 1997, **78**, 1396-1396.

47. C. Adamo and V. Barone, Toward reliable density functional methods without adjustable parameters: The PBE0 model, *J. Chem. Phys.*, 1999, **110**, 6158-6170.

48. S. E. Wheeler, A. Moran, S. N. Pieniazek and K. N. Houk, Accurate reaction enthalpies and sources of error in DFT thermochemistry for aldol, Mannich, and alpha-aminoxylation reactions, *J. Phys. Chem. A*, 2009, **113**, 10376-10384.

49. J. Tomasi, B. Mennucci and R. Cammi, Quantum mechanical continuum solvation models, *Chem. Rev.*, 2005, **105**, 2999-3093.

50. J. W. Turner, B. E. Hartman and P. G. Hatcher, Structural characterization of suberan isolated from river birch (*Betula nigra*) bark, *Org. Geochem.*, 2013, **57**, 41-53.

51. A. P. Deshmukh, A. J. Simpson, C. M. Hadad and P. G. Hatcher, Insights into the structure of cutin and cutan from *Agave americana* leaf cuticle using HRMAS NMR spectroscopy, *Org. Geochem.*, 2005, **36**, 1072-1085.

52. J. Graça and H. Pereira, Cork suberin: A glyceryl based polyester, *Holzforschung*, 1997, **51**, 225-234.

53. H. Pereira, Variability of the chemical composition of cork, *Bioresources*, 2013, **8**, 2246-2256.

54. J. P. Hallett and T. Welton, Room-temperature ionic liquids: Solvents for synthesis and catalysis. 2, *Chem. Rev.*, 2011, **111**, 3508-3576.

55. S. Chowdhury, R. S. Mohan and J. L. Scott, Reactivity of ionic liquids, *Tetrahedron*, 2007, **63**, 2663-2389.

56. C. C. Weber, A. F. Masters and T. Maschmeyer, Controlling hydrolysis reaction rates with binary ionic liquid mixtures by tuning hydrogen-bonding interactions, *J. Phys. Chem. B*, 2012, **116**, 1858-1864.

57. J. Restolho, J. L. Mata and B. Saramago, Choline based ionic liquids: Interfacial properties of RTILs with strong hydrogen bonding, *Fluid Phase Equilibr.*, 2012, **322-323**, 142-147.

Chapter VI

Biomimetic suberin as novel hydrophobic antimicrobial materials

Adapted from: H. Garcia, R. Ferreira, C. Martins, A. F. Sousa, C. S. R. Freire, A. J. D. Silvestre, W. Kunz, L. P. N. Rebelo and C. Silva Pereira, Biomimetic suberin as novel hydrophobic antimicrobial materials, *submitted manuscript*, 2013.

The author had contributed to the planning and execution of all the experiments herein presented, as well as on the discussion, interpretation and preparation of the manuscript. XRD, AFM, SEM, solid-state NMR, Zeta potential and the biological and microscopic analysis were performed in collaboration with technicians or co-authors.

1. Abstract

Biopolymers often have unique properties of considerable interest as basis for new materials. It is however not evident how to extract them from plants without destroying their chemical skeleton and inherent properties. Here we report the *ex-situ* reconstitution of suberin as a new hydrophobic and antimicrobial material. In plant cell walls suberin, a cross-linked network of aromatic and aliphatic monomers, builds up a hydrophobic protective and antimicrobial barrier. Recently we succeeded in extracting suberin from the plant cell wall in the ionic liquid cholinium hexanoate. During extraction the native three-dimensional structure of suberin was partially preserved. In this study, we demonstrate that this preservation is a key for *ex-situ* spontaneous reconstitution. Without any chemical additives or purification, the suberin composing macromolecules undergo self-association on the casting surface as films. Suberin films obtained show properties similar to the suberin barrier in plant including a potentially broad bactericidal effect.

2. Introduction

Polymers, particularly polyesters, are present in literally every aspect of modern life, from the more mundane to rather sophisticated applications. Up to now, most polyester-based materials still depend on petroleum-based chemistry. Yet polyesters exist in numerous renewable sources and commonly show unique chemical skeletons depending on the source. Differences in skeleton can translate into specific properties, including bio-compatibility and degradability, as well as biological activity. Both suberin and cutin have been in the centre of such efforts[1]. Biopolyesters are widespread in higher plants, deposited in the cell walls as a protective hydrophobic barrier[2,3]. They show high abundance of hydroxyacids and corresponding derivatives carrying mid-chain functionalities such as unsaturated, epoxy or *vic*-diol groups, rarely found in other polyester from other sources. They are prominent sources of unique building blocks for development of innovative materials[1]. Biomimetic materials built with hydroxyacids with mid-chain functionalities have been produced through polycondensation reactions and the

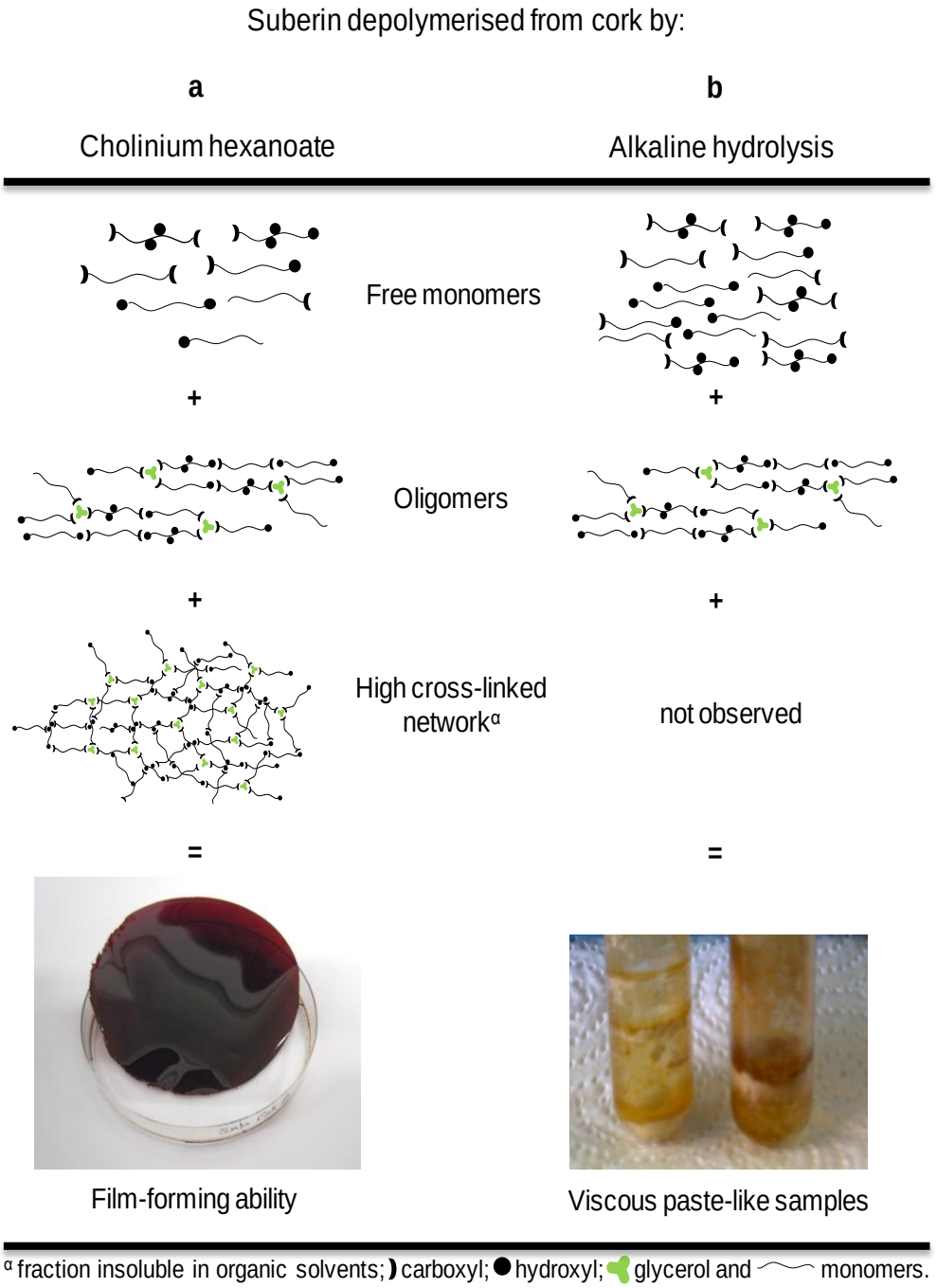


Figure 1| Schematic representation of suberin samples obtained from cork. Suberin depolymerisation through **a**, cholinium hexanoate and **b**, alkaline hydrolysis.

transesterification of either a mix of dissimilar monomers or of a single monomer upon purification[4-7].

Elegant progress has been made but so far the *ex-situ* reconstitution of a plant cell wall biopolyester as a material has never been demonstrated. Recent evidence that some cutin purified hydroxyacids undergo self-assembly[8] encouraged us to develop a material mimetic of the biopolyester cell wall barrier, in particular from suberin. Suberin offers strategic advantages over cutin due to its greater diversity of monomers together with its high abundance in *Quercus suber* L. cork (*ca.* 50%wt)[9]. The polyaliphatic domain of suberin also comprises, covalently linked essentially via ester bonds, a minor polyphenolic domain[1,9-11].

Suberin is a structural component of the plant cell wall. At least partial depolymerisation is necessary to efficiently isolate this biopolyester[2,3]. Typically, alkaline hydrolysis or methanolysis that non-specifically cleave ester bonds have been used, leading to the degradation of the suberin skeleton[1]. More recently we have proposed partial hydrolysis; acylglycerol ester bonds in suberin are efficiently cleaved, whilst linear aliphatic ester bonds are largely preserved[12,13]. This approach uses ionic liquid cholinium hexanoate, in the dual role of solvent and catalyst during suberin extraction from plant cell walls[14]. The macromolecular structure of this extracted suberin, which from now on we refer to as *ex-situ* suberin, retains aspects of the native structure. It is built essentially of oligomers of high molecular weight and cross-linked structures plus minor amounts of aromatic and free aliphatic monomers (Figure 1a). The cross-linked structures, which are insoluble in organic solvents, contain non-hydrolysable moieties.

3. Methods

3.1 Suberin film casting process

Suberin samples were extracted as previously described[14]. Briefly, cork was mixed with cholinium hexanoate during 4h at 100 °C, with stirring. The mixture was first filtered to remove solids, the precipitation of suberin in the filtrate was promoted by adding an excess of water (4 °C, overnight), which was then recovered by centrifugation. Suberin

films were produced by depositing a suspension of *ex-situ* suberin in water onto a polystyrene plate, followed by slow evaporation of water, first at 30 °C during 4 days and then at 50 °C for 5 days, until complete dryness. Suberin extraction using alkaline hydrolysis[15] and methanolysis with calcium hydroxide[16] was done as previously reported.

3.2 Spectroscopic analysis of suberin films

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). ATR-FTIR spectra were collected on a Bruker IFS66/S FTIR spectrometer (Bruker Daltonics, MA, USA) using a single reflection ATR cell (DuraDisk, equipped with a diamond crystal). Data were recorded at room temperature, in the range of 4000-600 cm^{-1}, by gathering 128 scans at a resolution of 4 cm^{-1}. Five replica spectra were collected for each sample in order to evaluate reproducibility (OPUS v5.0).

X-ray diffraction (XRD). XRD analysis was performed using a Philips X'pert MPD instrument operating with CuK_α radiation (λ = 1.5405980 Å) at 40 kV and 40 mA. Samples were scanned in the 2θ range of 3 to 70°, with a step size of 0.04°, and time per step of 60 s.

3.3 Wettability of suberin films

Water contact angle. Water contact angle measurements (OCA 20, Dataphysics) were performed at room temperature, on both surfaces of the suberin film. A 2 μL drop of distilled water was dispensed on the surface of each film using a microsyringe (Hamilton DS500/GT). Image analysis software (Dataphysics SCA20_M4) was used to calculate the contact angles of the drops by the Laplace-Young method. Six replicates were collected for each suberin surface.

Moisture uptake. Suberin films (dimensions 5 × 45 × 0.1 mm) were conditioned at room humidity of 33, 52 and 91%, achieved by maintaining the samples in closed atmospheric equilibrium at room temperature with saturated solutions of $MgCl_2$, $Mg(NO_3)_2$ and $BaCl_2$, respectively. Three samples were tested for each salt at room humidity. The weight increase due to water absorption was periodically assessed during a period of 21 days.

The water uptake, $wt_{H2O\ uptake}$, at time t was calculated as $wt_{H2O\ uptake} = 100 \times (m_t - m_{t=0})/m_{t=0}$ where m is the mass of the suberin film.

3.4 Mechanical and thermal properties of the suberin films

Thermal properties. Differential Scanning Calorimetry (DSC) analyses were carried out with a DSC – Q200 TA Instrument calibrated for temperature and heat flow with indium samples and operated under constant purging of nitrogen (50 cm^3 min^{-1}). Samples were hermetically sealed in aluminium pans and heated/cooled up to 120/- 80 °C at a constant rate of 5 °C min^{-1}, followed by a 5 min isotherm at 120/-80 °C. Three heating/cooling cycles were repeated. The first cycle was used to clear the sample thermal history. When second and third cycles were identical, the latter was used for data collection. Characteristic peaks were analysed using Universal Analysis, software version 4.4A. Melting temperature (T_m) was determined as the minimum of the melting endothermic peak during the heating cycle.

Mechanical properties. We used a texture analyser (model TA.Hdi, Stable Micro Systems) equipped with a 5 Kg cell and fixed clamps covered with rubber. First mean thickness of the film, an average of five measurements at different points, was calculated using a digital micrometre (model MSC-25S, Mitutoya Crop., Tokyo, Japan, precision 0.001 mm). Film strips (70 mm long, 10 mm wide) were pressed by clamps using an initial grip separation of 50 mm, then their mechanical resistance was tested using a crosshead speed of 0.1 mm/s (eleven replicates). Young's modulus (E), percentage elongation at break (ε_R) and tensile strength or maximum stress (σ_B) were determined from stress-strain curves from uniaxial tensile tests to film failure. All experiments were conducted at room conditions (RT=18°C and RH=33%).

3.5 Antimicrobial assays of suberin films

The antimicrobial dynamic contact tests against the bacteria *Staphylococcus aureus* NCTC8325 and *Escherichia coli* TOP 10 were conducted following the ASTM E2149-01 guideline[17]. Bacterial cultures (50 mL, Müller-Hinton Broth) were incubated in the presence of suberin film pieces (4 mg/mL) and the number of colony forming units

(CFUs) monitored at time points 0h, 2h, 4h, 8h, 12h and 24h by spreading an aliquot of the culture (10 μL) onto solid media (Plate Count Agar). Control cultures (without the suberin film) were also prepared. The results were expressed as follows:

$$\% \text{ kill} = [(CFU_{CONTROL} - CFU_{TEST}) \times 100] / CFU_{CONTROL}$$

To evaluate bacterial viability during exposure to the suberin films (see above), aliquots (1 ml) of the culture were collected at different incubation time points and the cells labelled with propidium iodide and fluorescein diacetate (15 minutes, room temperature, with agitation)[18,19]. Bacterial cells were observed by phase contrast and fluorescence microscopy with a DM5500 B fluorescence microscope (Leica) using a 49 DAPI and N21 filter sets, a 100x magnification objective and images captured with an Andor Luca R EMCCD camera. The micrographs presented here are representative.

3.6 Surface characterisation of suberin films

Microscopy. Phenolic and aliphatic suberin on the surface of the films was detected by microscopy using a 100x magnification objective (see above). Fluorescence microscopy was used to search for aromatic groups on the surface of the films (auto-fluorescent under the UV light) and light microscopy was used to detect the aliphatic groups of suberin upon staining films with Sudan IV (15 minutes, without agitation), as previously described[20]. Micrographs presented here (captured as described above) are representative.

Atomic Force Microscopy (AFM). Tapping mode AFM experiments were performed in a multimode AFM microscope coupled to a Nanoscope IIIa, using a tip Tap300AI-G (BudgetSensors), with a frequency of ca. 300 KHz and a spring constant of 40 N/m, at room temperature and humidity. Scanning speed was optimized to 1.0 Hz and acquisition points were 512 × 512. Height, amplitude, phase and Zsensor were the acquisition channels used with an area of 10 μm, 3 μm and 1 μm (with a resolution of ca. 20nm, 6nm and 2nm, respectively). Imaging data were analyzed with the Gwyddion 2.31.

Zeta potential. The zeta potential of suberin films were determined by electrophoretic mobility measured in distilled water by means of a Zeta-Meter 3.0 +.

4. Results and Discussion

Suberin obtained from plant material using conventional extraction methods is a viscous paste-like material devoid of film-forming abilities[1]. Here, we demonstrate that the suberin depolymerised in the ionic liquid media can be reconstituted as a water-proof, hydrophobic and bactericidal film. These properties are similar to the suberin barrier in plant. Upon depolymerisation in cholinium hexanoate media, *ex-situ* suberin was recovered simply by promoting its precipitation in water[14] and used without further purification. Suberin films were produced by directly casting a diluted aqueous suspension of *ex-situ* suberin on the surface of a polystyrene plate (Figure 1a). Water was removed by evaporation, first at 30 ºC and then at 50 ºC. Production of this biopolyester film requires neither additives nor chemical synthesis. Importantly, the film-forming ability was conserved in *ex-situ* suberin samples prepared by freeze-drying of the suspension at -20 ºC, which from now on we refer to as *ex-situ* suberin powder. Suberin films present a plastic consistency and are stable, non-leaching after immersion in an aqueous media for at least six months.

The suberin films produced here proves for the first time that biopolyesters can be reconstituted *ex-situ* as a film through the self-association of the composite macromolecules providing the native structure is partially preserved. Given at least partial structural preservation, the composing macromolecules can effectively attract each other, either by hydrogen bonding interactions or hydrophobic interactions. Suberin films and powders had virtually identical ATR-FTIR (Figure 2; see Supplementary Section S3.2 for further details) and ^{13}C CP/MAS NMR spectra (Supplementary Section S1). They are in turn equivalent to those previously reported by us in dried *ex-situ* suberin samples[14,21], suggesting no covalent bonds were created between the composing macromolecules during the formation of the film. Additional experiments support the idea that the partial preservation of the native macromolecular structure ensured film forming abilities of the *ex-situ* suberin. Suberin extracted from cork using alkaline hydrolysis which cleaves extensively ester bonds (Figure 2, note peak at 1706 cm^{-1} representative of acid moieties), does not form films (Figure 1b). The last step of this

alkaline hydrolysis is the partitioning of the sample in an organic solvent, discarding the high cross-linked fraction. Even when this step was eliminated to prevent segregation of the sample between the two phases we could not form films. We then used methanolysis with calcium hydroxide, which non-extensively cleaves ester bonds[16]. This partially depolymerised suberin sample (Figure 2) was also viscous and lacking in film-forming ability. Solvent partition of the *ex-situ* suberin also obstructed its film-forming ability. With partition in the organic solvent, the insoluble fraction was discarded, *i.e.* high cross-linked structures with approximately 44%wt (Figure 1a). Water plays the role of anti-solvent during the precipitation of the *ex-situ* suberin after its depolymerisation in cholinium hexanoate and during casting. This avoids differential segregation of the composing macromolecules, ensuring film forming abilities of the *ex-situ* suberin.

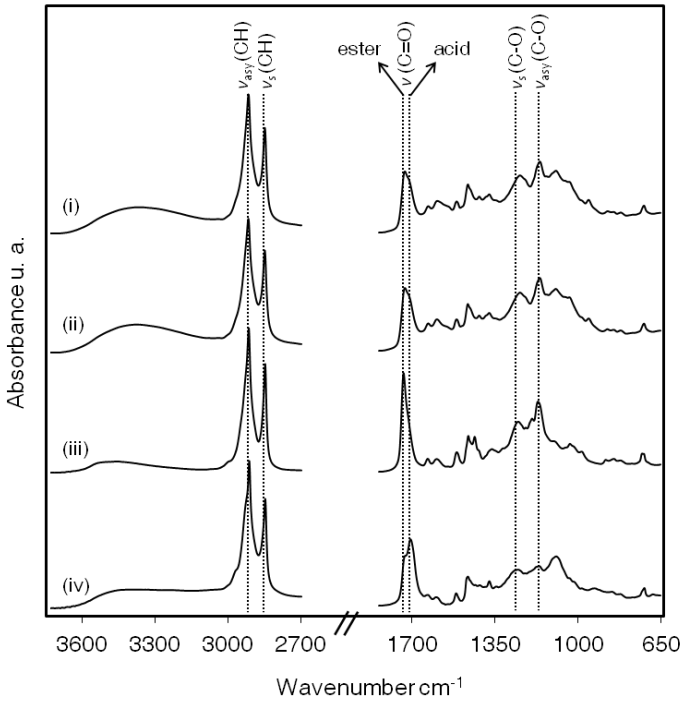


Figure 2| Characterisation of suberin samples by ATR-FTIR spectroscopy. Spectral features of: (i) suberin film produced with *ex-situ* suberin (depolymerised with cholinium hexanoate), (ii) suberin powder prepared by freeze-drying of *ex-situ* suberin suspensions, (iii) suberin samples prepared by conventional methanolysis with calcium hydroxide and (iv) suberin samples prepared by conventional alkaline hydrolysis with sodium hydroxyde.

Glycerol is a key cross-linker in the formation of a three-dimensional network, connecting hydrophilic moieties and both suberin domains[10]. *Ex-situ* suberin recovered after 4h of hydrolysis catalysed by the ionic liquid contains only *ca.* 8 mg hydrolysable glycerol *per* g suberin (*i.e.* 10%wt of the total hydrolysable glycerol)[13]. To clarify the role of glycerol we also prepared suberin samples where all the glycerol had already been released (8 h). The 8 h sample showed a lower fraction of cross-linked structures, thus higher solubility in dichloromethane, than the 4 h samples (65%wt and 56%wt, respectively). Most ester bonds different from an acylglycerol ester bond were probably preserved in 4h and 8h samples[13]. Both samples were composed principally of high molecular weight oligomers and cross-linked structures, yet the 8 h sample produced extremely brittle films that were presenting extensive cracks (Supplementary Section S2). Fine tuning of ester bond hydrolysis by the ionic liquid catalyst is therefore vital for film-forming ability of the *ex-situ* suberin. This underlines the importance of the mild plus specific depolymerisation, which so far can only be attained using an ionic liquid.

Most *ex-situ* suberin is composed of high molecular weight and cross-linked structures, raising the possibility that the reconstitution as a film of this biopolyester is essentially unstructured. We observed that suberin films generated were essentially amorphous. They display the typical X-ray diffraction pattern with a broad amorphous halo (centred *ca.* $2\theta \approx 20^{\circ}$) (Figure 3a). They also showed a broad melting transition,

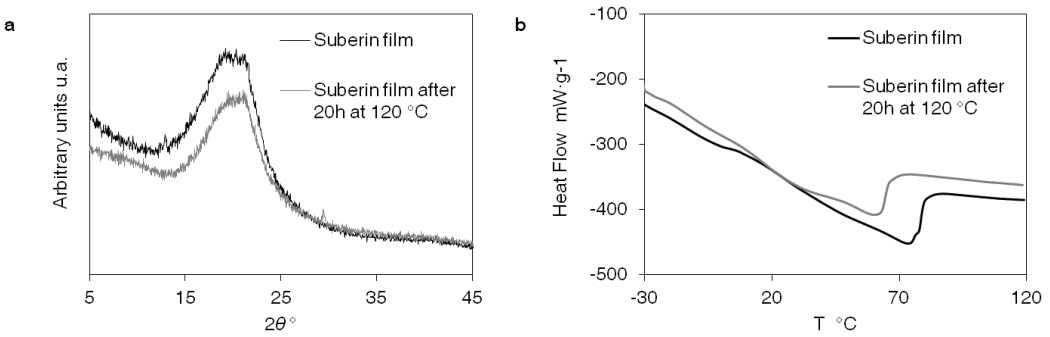


Figure 3| X-ray and thermal characterisation of suberin films. Suberin film and suberin film treated during 20h at 120 °C were characterised by X-ray diffraction pattern (**a**) and DSC analysis (**b**).

spanning from 30 to 70 °C in the DSC thermogram (Figure 3b), indicative of some crystalline domains. The DSC thermogram of the suberin film is virtually identical to that of the *ex-situ* suberin powder, reinforcing the idea that the film forms essentially due to self-association of the suberin macromolecules. After 20 h at 120 °C, films were still amorphous (Figure 3a), displayed lower solubility in an organic solvent (1.5 fold reduction) and a more restricted melting transition temperature in the DSC thermogram (Figure 3b). Apparently this treatment induced cross-linking, rather than a higher degree of structural organisation as could be inferred from the DSC data alone. One can hypothesise that hydroxyacids and dicarboxylic acids with unsaturated bonds and epoxy rings existing in the suberin films grant properties similar to other thermosetting biomaterials (*e.g.* castor and soybean oils)[22]. This might prove useful since the films, as predicted, were narrowly elastic and relatively brittle (Young's modulus of 63.2 ± 1.8 MPa, a tensile strength of 1.9 ± 0.1 MPa and an elongation at break 6.7 ± 0.6 %). Films were unable to accommodate substantial deformation as the stress imposed irreversibly affected their three-dimensional network. Cutin materials have been isolated from the fruit cuticles by removing the cell wall polysaccharides through enzymatic digestion[23]. These materials (which are chemically related to suberin films) have been also found to exhibit low elasticity and to be essentially amorphous, whilst preserving some crystalline domains[7,24].

We investigated whether suberin films can act as a water barrier, since suberin in the cell wall forms a barrier which prevents the uncontrolled loss of water and nutrients from plants[3]. They proved to be impermeable to water when using a vacuum filtration device. We then determined the wetting kinetics of the suberin films; both suberin film surfaces can be considered hydrophobic since after 500 ms, usually a sufficient time-scale for a thermodynamic equilibrium to be reached[25], the water contact angle was *ca.* 90°. Suberin films were found to be wettable, albeit with very limited water uptake capacity, 3%wt, 5%wt and 7%wt in a controlled atmosphere of 33%, 52% and 91% humidity, respectively. The spread water on the suberin film surface was rapidly lost when the films were again exposed to low humidity environments. Water drop spreading was also observed; at longer contact periods, the contact angle of water decreased in both surfaces.

After 15 s, it reached *ca.* 65° and was steady up to 90 s. Similar behaviour has been reported for the flat and non-porous surface of an alkaline hydrolysed suberin sample dried over glass or polyethylene[25].

The cell wall suberin barrier is not only virtually impermeable to water and solutes, but is also resistant to microbial hydrolysis and has antimicrobial activity[3,26]. The aromatic moieties confer suberin with antimicrobial features, typical of phenolics. The aliphatic domain physically strengthens the cell wall, protecting it from microbial hydrolysis, and may also exhibit antimicrobial properties[20]. Here we demonstrate that after spontaneous reconstitution of *ex-situ* suberin as a film, we preserve the antimicrobial properties of the native suberin. Under dynamic contact conditions suberin films are bactericidal both against Gram- positive *Staphylococcus aureus* and negative *Escherichia coli*. When grown in the presence of the suberin film (4 mg/mL) a dramatic reduction in the number of viable bacteria was rapidly detected. Bactericidal effect (% of kill) was inferred through comparison to the corresponding controls (bacteria grown in broth media without the suberin film). Bacterial death was noticed along the entire growth-curve (Figure 4a), yet more accentuated when the culture reached the stationary phase of growth; after12 h of incubation, growth inhibition was 99.9% and 94.3% for the Gram-positive and the Gram-negative bacteria, respectively (Figure 4a). The number of viable bacteria in the control cultures decreased between the twelfth hour and the twenty-fourth hour. Growth inhibition values reached 98.4% and 94.2% for the two bacterial species (Figure 4a). The bactericidal effect of suberin films was further confirmed by fluorescent microscopy using a LIVE/DEAD assay (Figure 4b-e). The number of dead bacteria largely exceeded that of the live bacteria. Neither tested bacteria was able to degrade the suberin film in any meaningful way; surface composition and morphology were unaltered (Supplementary Section S3). No adhesion of bacteria on the surface of the film was detected, suggesting that the film reduces biofouling.

The anti-biofouling effect might be related to surface topography[27], suggesting that the suberin films might provide a smooth surface. We have used AFM to collect topographic images (tapping mode) of suberin films, both of the superior and inferior surfaces (Figure 4f-k). They show a very low degree of roughness (R_a of 0.024

μm - superior - and 0.002 μm - inferior). They can be classified as smooth or sliding surfaces, which usually are associated with inhibition of cell adhesion. Film surfaces show also a heterogeneous arrangement of "spheroid bumps" (average diameter of 0.41 ± 0.12 μm), some of which are randomly scattered while others overlap. As expected, the film repulsed the AFM tip. Repulsion was slightly more pronounced in regions surrounding the bumps, typical of hydrophobic surfaces. Some bumps had concentrically aligned layers spaced a few nm (*ca.* 5 to 10 nm), remarkably similar to the lamellar organisation of the polyaliphatic domain of suberin of alternate aliphatic and phenolic (*viz.* hydroxycinnamates) components[2,11]. It suggests that some recalcitrant fractions of the suberin lamella where conserved (non-hydrolysable domains), reinforcing the idea that *ex-situ* suberin preserved at least partially the native structure.

Long aliphatic cutin monomers have been suggested to undergo self-assembly on hydrophobic casting surfaces following a layered pattern in which molecules align[8]. Despite the higher complexity of suberin the polyaliphatic domain is composed mainly of dicarboxylic and hydroxyacids derived from C_{16} to C_{26}[1,9-11]. It is likely that some long aliphatic chains had self-associated in a similar mode during spontaneous reconstitution of the *ex-situ* suberin as a film. Such alignment agrees with the poor electrostatic potential of the film surface as defined by their Zeta potential (ζ = -19.0 mV ± 2.6). The slight negativity of the surface probably derived from the few long aliphatic chains with acidic end groups at the film surface. Long-chain acids and/or alcohols have been postulated to interact strongly with bacterial cell boundaries, leading to their disruption and death[28]. Poor electrostatic potential also agrees with the idea of an anti-biofouling surface[29]. After Sudan IV staining, a dye commonly used to detect lipids and triglycerides[20], most of the surface of the suberin film had a red tinge (Figure 4l), revealing, as predicted, the presence of aliphatic suberin. Despite relatively low levels of hydroxycinnamic acid, its derivatives and monolignols in suberin[11], it has been repeatedly postulated that these compounds are responsible for the antibacterial effect of suberin[20,26]. The association of such polar groups at the surface of the suberin film during the casting in water is also possible. Few phenolic compounds, which auto-fluoresce in the UV

range, were detected at the surface of the film (Figure 4m). It appears that the film's bactericidal effect involves both aliphatic and aromatic compounds.

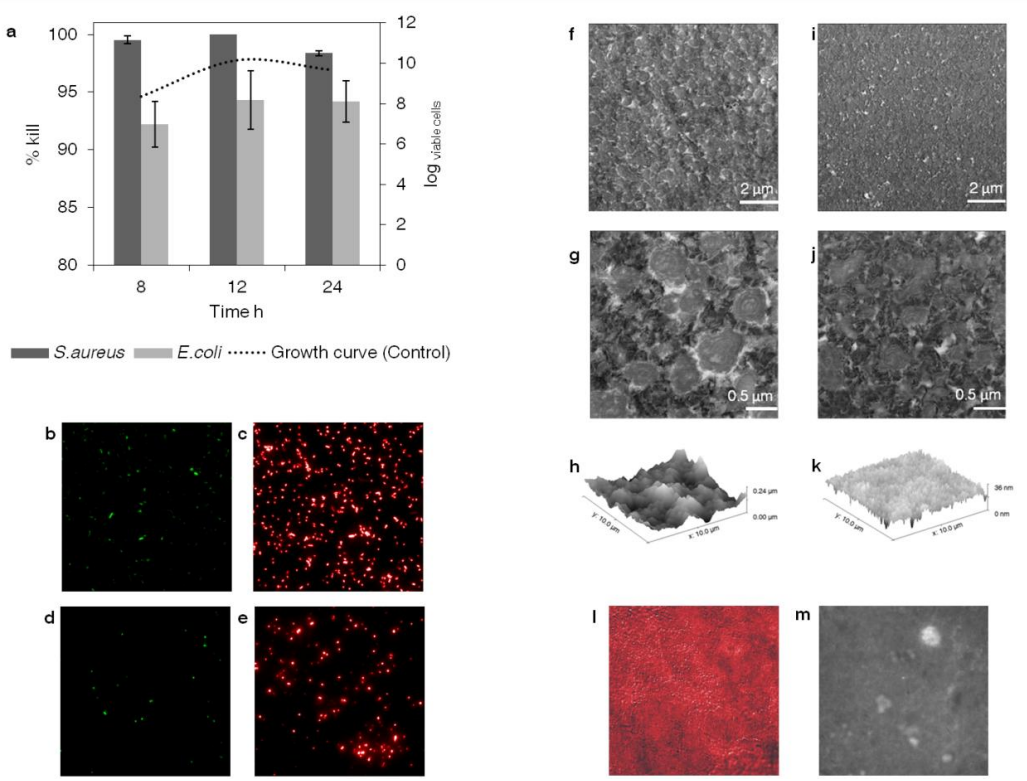


Figure 4| Antimicrobial activity against bacteria and surface characterisation of the suberin film. a, % Kill of *E. coli* and *S. aureus* on suberin films (principal vertical axis) and Log_{10} of viable cell in control cultures (secondary vertical axis). **b-e,** LIVE/DEAD bacterial viability assay of *E. coli* (**b,c**) and *S. aureus* (**d,e**) after 12h. **f-k,** AFM images of the superior (**f-h**) and inferior (**i-k**) surfaces of the suberin films acquired with the channels: amplitude (**f,i**), phase (**g,j**) and height (**h,k**). **l,m,** Histochemical characterisation of the suberin film surface using Sudan IV stain for aliphatic compounds (**l**) and auto-fluorescence of aromatic compounds (**m**).

5. Conclusion

In the present contribution we for the first time demonstrated that plant polyesters can be reconstituted *ex-situ* as films, in particular suberin films. We showed that the film forms because the native structure of suberin was partially preserved during its extraction from

the plant cell wall. This was achieved using cholinium hexanoate which acts simultaneously as solvent and mild plus selective catalyst, promoting almost exclusively the cleavage of acylglycerol esters bonds. Whilst glycerol is the key cross-linker in suberin, preservation of linear aliphatic ester bonds uniting different layers of aliphatic suberin secured the partial preservation of the native structure. This, fundamental for the spontaneous reconstitution of the *ex-situ* suberin as a film, might explain partially the film's bactericidal properties. We confirmed that suberin films are mimetic of the suberin barrier in plant cell walls. They are hydrophobic and water-proof and show antimicrobial and anti-biofouling properties. This study should inspire development of other biopolyester-based materials for a broad range of applications. One of the first applications we believe will be implemented is clinical usage, also due to the biocompatibility of suberin films.

6. Acknowledgements

R.F. and A.F.S are grateful to Fundação para a Ciência e a Tecnologia (FCT), Portugal, for the fellowships SFRH/BD/48286/2008 and SFRH/BPD/73383/2010, respectively. H.G. is indebted to Fundação Calouste Gulbenkian, Portugal, for the fellowship 21-95587-B. The work was partially supported by a grant from Iceland, Liechtenstein and Norway through the EEA financial mechanism (PT015), by FCT through the grants PEst-OE/EQB/LA0004/2011, Pest-C/CTM/LA0011/2011, PTDC/QUI-QUI/120982/2010, PTDC/AAC-CLI/119100/2010 and REDE/1517/RMN/2005, and by a cooperation grant from FCT and the Deutscher Akademischer Austauschdienst (DAAD). The authors thank Dr. M. Matzapetakis (ITQB-UNL, PT) and Dr. R. Machado (IST-UTL, PT) for helping in the acquisition of NMR and Zeta potential data, respectively, and Professor H. Lencastre and Dr. J. Mota, for providing the bacterial strains used in this study. We are thankful to *peer*-colleagues who read and critically commented on the final manuscript.

7. Supplementary Information

Section 1 (S1)| NMR spectroscopic characterisation of suberin film and *ex-situ* suberin powder

S1.1 – Methods

^{13}C Cross Polarization Magic/Angle Spinning NMR (CP/MAS NMR). The solid state spectra of the *ex-situ* suberin powder and the *ex-situ* suberin were collected on a Bruker Avance II+ 800 NMR spectrometer operating at 800.33 MHz for proton and 201.24 MHz for carbon, using a TriGama MAS 3.2mm probe with the sample rotating at 12KHz. The *ex-situ* suberin samples were finely powdered while mixed with liquid N_2 and then dried, while the powdered *ex-situ* suberin was used as-is. A proton 90^o pulse of 5μs, a CP contact time of 1ms and a recycle delay of 2s were used to accumulate 10ms of data points for each sample using 12k scans.

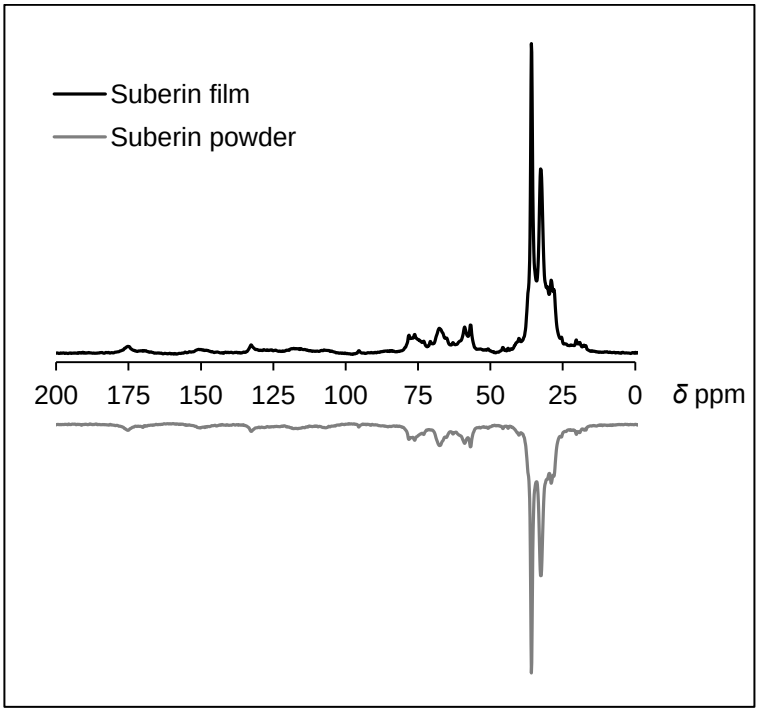


Figure S1| ^{13}C CP/MAS NMR spectra of the suberin film and the *ex-situ* suberin powder.

S1.2 - Results

The ^{13}C CP/MAS NMR spectra of the suberin film and the *ex-situ* suberin powder showed that both samples own an essential aliphatic and esterified nature. In detail, both suberin samples are characterised by the dominance of aliphatic signals (CH_2: δ 30/33 ppm) and also the presence of ester moieties (C=O: δ 173 ppm and C-O: δ 64 ppm) as a result of the mild depolymerisation promoted by cholinium hexanoate. Other resonances assigned to carbons nearby hydroxyl or ester groups are also present (δ 54, 64, 73 ppm). Typical aromatic signals from suberin were also identified (C=C: δ 130 ppm and 1635 cm^{-1}), although with low intensity. The present data further validate the ATR-FTIR analysis of the suberin film (see Supplementary Section S3.2 for further details).

Section 2 (S2)| Suberin film produced with a suberin sample recovered after 8h of extraction from cork with cholinium hexanoate

S2.1 - Methods

Suberin films cast process was conducted as described in the Main Text. These suberin samples were extracted from cork with cholinium hexanoate during 8h at 100 °C with stirring.

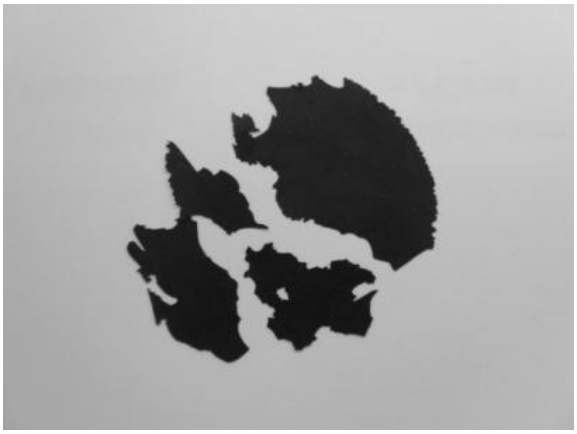


Figure S2| Film prepared with suberin obtained after 8 h of cork depolymerisation with cholinium hexanoate.

Section 3 (S3)| ATR-FTIR spectroscopic analysis and SEM morphologic characterisation of suberin films after incubation with bacteria.

S3.1 - Methods

Attenuated total reflectance - Fourier transform infrared spectroscopy (ATR-FTIR). The suberin films were collected after incubation with bacteria, dried and analysed by ATR-FTIR as described in the Main Text.

Scanning electron microscopy (SEM). The suberin films were collected after incubation with bacteria. Samples were dried prior to use and coated with a thin layer of gold using a sputter coater (Polaron E-5100). Electron micrographs were recorded using an analytical field emission gunscanning electron microscope (FEG-SEM: JEOL 7001F with Oxford light elements EDS detector) operated at 5–10 kV. The micrographs presented here are representative of the different fractions.

S3.2 - Results

The suberin films were collected after incubation with bacteria, namely *Staphylococcus aureus* (Figure S3ii) or *Escherichia coli* (Figure S3iii), and analysed by ATR-FTIR spectroscopy. Both spectra showed to be identical to the spectrum of an untreated suberin film (Figure S3i). This observation suggests that none of the tested bacteria was able to degrade the suberin films.

All the ATR-FTIR spectra showed to be dominated by two major peaks (ν_{ass} C-H 2920 cm^{-1} and ν_s C-H 2851 cm^{-1}), mostly attributed to the long aliphatic chains of suberin. The polyester nature of the suberin films were further confirmed by the presence of ester moieties (ν C=O 1735 cm^{-1} and ν C-O-C 1164/1245 cm^{-1}). In addition, typical hydroxyl and aromatic signals of suberin are also identified (ν O-H 3679-3034 cm^{-1} and ν C=C 1635 cm^{-1}, respectively).

The morphological surface of the suberin films, after their contact with the selected bacteria, were further analysed by SEM (Figure S4). Again, both suberin films presented no morphological alterations relatively to the untreated suberin film. In addition, no adhesion of bacteria on the surface of the both films was detected. These

observations further reinforce the idea that none of the tested bacteria was able to degrade the suberin film, since its surface composition and morphology were unaltered.

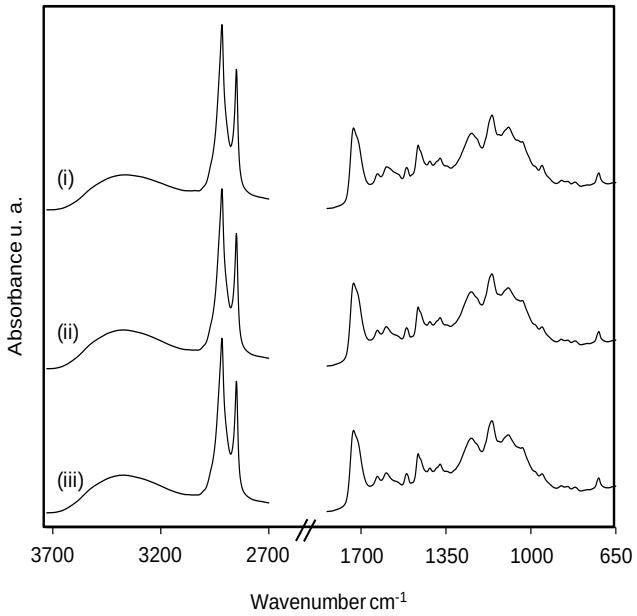


Figure S3| Characterisation of suberin samples by ATR-FTIR spectroscopy. Spectral features of suberin films: (i) untreated, (ii) after incubation with *S. aureus* and (iii) after incubation with *E. coli*.

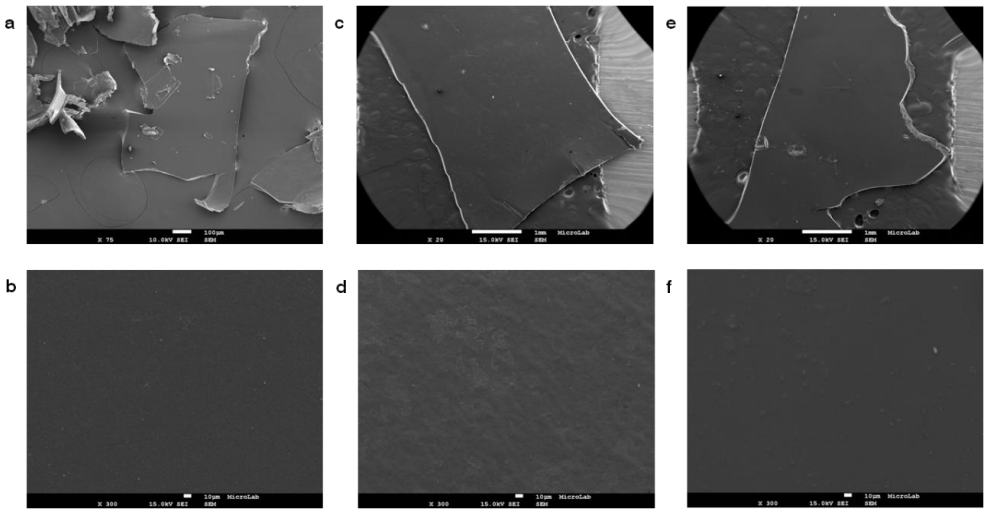


Figure S4| SEM images of suberin films: a,b untreated, **c,d** after incubation with *E. coli* and **e,f** after incubation with *S. aureus*.

8. References

1. A. Gandini, C. Pascoal Neto and A. J. D. Silvestre, Suberin: A promising renewable resource for novel macromolecular materials, *Prog. Polym. Sci.*, 2006, **31**, 878-892.
2. P. E. Kolattukudy, Biopolyester membranes of plants: Cutin and suberin, *Science*, 1980, **208**, 990-1000.
3. L. Schreiber, Transport barriers made of cutin, suberin and associated waxes, *Trends Plant Sci.*, 2010, **15**, 546-553.
4. A. Olsson, M. Lindström and T. Iversen, Lipase-catalyzed synthesis of an epoxy-functionalized polyester from the suberin monomer *cis*-9,10-epoxy-18-hydroxyoctadecanoic acid, *Biomacromolecules*, 2007, **8**, 757-760.
5. A. F. Sousa, A. Gandini, A. J. D. Silvestre and C. Pascoal Neto, Synthesis and Characterization of Novel Biopolyesters from Suberin and Model Comonomers, *ChemSusChem*, 2008, **1**, 1020-1025.
6. A. F. Sousa, A. Gandini, A. J. D. Silvestre, C. Pascoal Neto, J. J. Cruz-Pinto, C. Eckerman and B. Holmbom, Novel suberin-based biopolyesters: From synthesis to properties, *J. Polym. Sci. Pol. Chem.*, 2011, **49**, 2281-2291.
7. J. A. Heredia-Guerrero, A. Heredia, R. García-Segura and J. J. Benítez, Synthesis and characterization of a plant cutin mimetic polymer, *Polymer*, 2009, **50**, 5633-5637.
8. J. A. Heredia-Guerrero, M. A. San-Miguel, M. S. P. Sansom, A. Heredia and J. J. Benítez, Chemical Reactions in 2D: Self-assembly and self-esterification of 9(10),16-dihydroxypalmitic acid on mica surface, *Langmuir*, 2009, **25**, 6869-6874.
9. P. E. Kolattukudy, Polyesters in higher plants, in: *Biopolyesters*, ed. T. Scheper, W. Babel and A. Steinbuchel, 2001, vol. **71**, pp. 1-49.
10. H. Pereira, Cork: biology, production and uses, *Elsevier*, Amsterdam, The Netherlands, 1st edn., 2007.
11. M. A. Bernards, Demystifying suberin, *Can. J. Bot.-Rev. Can. Bot.*, 2002, **80**, 227-240.
12. H. Garcia, R. Ferreira, M. Petkovic, J. L. Ferguson, M. C. Leitão, H. Q. N. Gunaratne, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Dissolution of cork biopolymers in biocompatible ionic liquids, *Green Chem.*, 2010, **12**, 367-369.
13. R. Ferreira, H. Garcia, A. F. Sousa, M. Guerreiro, F. J. S. Duarte, C. S. R. Freire, M. J. Calhorda, A. J. D. Silvestre, W. Kunz, L. P. N. Rebelo and C. Silva Pereira, Ionic liquids as new catalysts for the hydrolysis of acylglycerol bonds: Suberin depolymerisation in cholinium hexanoate media, *submitted*, 2013.
14. R. Ferreira, H. Garcia, A. F. Sousa, M. Petkovic, P. Lamosa, C. S. R. Freire, A. J. D. Silvestre, L. P. N. Rebelo and C. Silva Pereira, Suberin isolation from cork using ionic liquids: Characterisation of ensuing products, *New J. Chem.*, 2012, **36**, 2014-2024.
15. A. F. Sousa, P. C. R. O. Pinto, A. J. D. Silvestre and C. Pascoal Neto, Triterpenic and other lipophilic components from industrial cork byproducts, *J. Agr. Food Chem.*, 2006, **54**, 6888-6893.
16. J. Graça and S. Santos, Linear aliphatic dimeric esters from cork suberin, *Biomacromolecules*, 2006, **7**, 2003-2010.
17. ASTM, in *ASTM International, West Conshohocken, PA*, 2001.
18. V. G. Correia, V. D. Bonifácio, V. P. Raje, T. Casimiro, G. Moutinho, C. L. da Silva, M. G. Pinho and A. Aguiar-Ricardo, Oxazoline-based antimicrobial oligomers: synthesis by CROP using supercritical CO_2, *Macromol. Biosci.*, 2011, **11**, 1128-1137.
19. L. Boulos, M. Prévost, B. Barbeau, J. Coallier and R. Desjardins, LIVE/DEAD® BacLight™: Application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water, *J. Microbiol. Meth.*, 1999, **37**, 77-86.

20. E. C. Lulai and D. L. Corsini, Differential deposition of suberin phenolic and aliphatic domains and their roles in resistance to infection during potato tuber (*Solanum tuberosum* L.) wound-healing, *Physiol. Mol. Plant P.*, 1998, **53**, 209-222.
21. R. Ferreira, H. Garcia, A. F. Sousa, C. S. R. Freire, A. J. D. Silvestre, L. P. N. Rebelo and C. Silva Pereira, Isolation of suberin from birch outer bark and cork using ionic liquids: A new source of macromonomers, *Ind. Crop. Prod.*, 2013, **44**, 520-527.
22. J.-M. Raquez, M. Deléglise, M.-F. Lacrampe and P. Krawczak, Thermosetting (bio)materials derived from renewable resources: A critical review, *Prog. Polym. Sci.*, 2010, **35**, 487-509.
23. J. J. Benítez, A. J. Matas and A. Heredia, Molecular characterization of the plant biopolyester cutin by AFM and spectroscopic techniques, *J. Struct. Biol.*, 2004, **147**, 179-184.
24. G. López-Casado, A. J. Matas, E. Domínguez, J. Cuartero and A. Heredia, Biomechanics of isolated tomato (*Solanum lycopersicum* L.) fruit cuticles: The role of the cutin matrix and polysaccharides, *J. Exp. Bot.*, 2007, **58**, 3875-3883.
25. N. Cordeiro, P. Aurenty, M. N. Belgacem, A. Gandini and C. Pascoal Neto, Surface properties of suberin, *J. Colloid Interf. Sci.*, 1997, **187**, 498-508.
26. K. Ranathunge, L. Schreiber and R. Franke, Suberin research in the genomics era—New interest for an old polymer, *Plant Sci.*, 2011, **180**, 399-413.
27. W. Teughels, N. Van Assche, I. Sliepen and M. Quirynen, Effect of material characteristics and/or surface topography on biofilm development, *Clin. Oral Implan. Res.*, 2006, **17**, 68-81.
28. A. Muñoz-Bonilla and M. Fernández-García, Polymeric materials with antimicrobial activity, *Prog. Polym. Sci.*, 2012, **37**, 281-339.
29. J. Azeredo, J. Visser and R. Oliveira, Exopolymers in bacterial adhesion: interpretation in terms of DLVO and XDLVO theories, *Colloid Surface B*, 1999, **14**, 141-148.

Chapter VII

Concluding remarks & Thesis impact

1. SWOT analysis

Suberin, at the heart of this thesis, was the source of inspiration for the development of a new biomaterial, which preserves some of its physical, chemical and biological native properties. The experimental approaches to achieve this main goal included the development of a new depolymerisation methodology based on benign ionic liquids. The following lines provide a critical evaluation of the work herein performed highlighting the strengths, weaknesses, opportunities and threats (SWOT analysis, Fig. 1) of this scientific contribution.

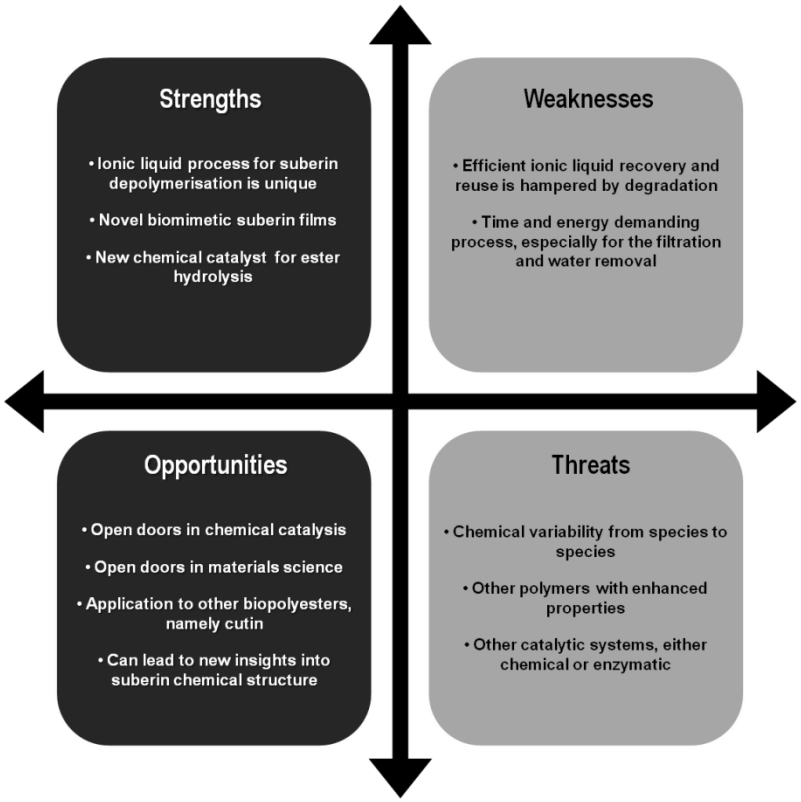


Figure 1| SWOT analysis of the work developed in the present dissertation.

A class of benign ionic liquids, namely the cholinium alkanoates (synthesised and characterised by our group[1]), were proved to efficiently depolymerise and isolate suberin from cork. This evidence constituted a remarkable advance in the ionic liquids field, since

it was the first demonstration that biocompatible and biodegradable ionic liquids were also able to dissolve plant biomass. Until then, imidazolium-based ionic liquids were the mostly used to deconstruct[2] and/or solubilise[3] biomass. The scope of possible applications using cholinium alkanoates may be favoured when compared to the use of imidazolium-based ionic liquids, since the last present high toxicity[4] and recalcitrancy towards biodegradation[5].

The results herein obtained further highlight cholinium hexanoate as the most promising ionic liquid to carry out the efficient separation of suberin at laboratory scale. The biopolyester was isolated with a straightforward optimised process through (1) mixture of cork with cholinium hexanoate during 4h at 100 °C, (2) filtration of the ensuing mixture to separate the cork insoluble residues from the filtrate and (3) addition of water to the filtrate in order to precipitate suberin, which was recovered by centrifugation and dried prior to characterisation. The depolymerised suberin showed to be mainly composed of oligomeric and cross-linked structures, revealing the partial preservation of its structure. Even though, the composing monomeric units were similar to those usually identified in suberin samples obtained by conventional depolymerisation processes[6-8].

The developed ionic liquid process allows, for the first time, the complete removal of suberin from cork through partial depolymerisation. On the contrary, common mild depolymerisation procedures, such as methanolysis with calcium hydroxide or calcium oxide, result in low extraction yields.([9] and references therein) These procedures, which aim at the preservation of the *in-situ* suberin, have been carried out mostly for suberin structural elucidation. Hence, this disruptive ionic liquid mild depolymerisation might well contribute, if carefully planned, to reveal important insights regarding suberin *in-situ* structure. Accordingly, this process has been already applied in samples collected from a time-course experiments of wound-healing potato tissues through collaboration with Professor Ruth Stark and her student Nancy Medina (CUNY).

To date, reports on hydrolysis catalysed by ionic liquids are mostly associated with the cleavage of ether bonds in lignocellulosic biomass.[10] In this study, the ionic liquid acted as a good solvent and catalyst promoting the efficient hydrolysis of

acylglycerol ester bonds, with those at C2 position reporting a faster kinetics, whilst most of the linear aliphatic esters remained intact. In addition, cholinium hexanoate showed to be unable to cleave the linkages of the other cell wall components, namely the glycosidic bonds of polysaccharides and the ether/C-C bonds present in lignin. These evidences reinforced the ionic liquid selectivity towards suberin. The present study also constitutes one of the few reports[11,12] of ionic liquids as efficient catalysts for the hydrolysis of esters. Notwithstanding the importance of other catalytic systems, this new chemical reaction highlights the large spectrum of ionic liquids application. Ionic liquids chemistry opens the possibility to design a catalyst displaying high selectivity towards a specific carbon centre in an ester bond.

Better understanding of the suberin depolymerisation mechanism revealed that the fine tuning of the ester bonds cleavage, and therefore the cross-linking degree, is essential for suberin *ex-situ* reconstitution as a film. To date, this specific partial preservation of the suberin *in-situ* structure can only be attained using ionic liquids. Suberin films, herein reported for the first time, were prepared directly after the depolymerisation, without any chemical modification or purification. Moreover, this new biomaterials retain some of the suberin barrier properties in plant cell walls. They are moderately hydrophobic, water-proof and show antimicrobial and anti-biofouling properties. The present study constitutes the proof-of-concept for the development of biopolyester films through a novel mild depolymerisation process using benign ionic liquids.

Besides its ability to depolymerise suberin from cork, cholinium hexanoate was also proved to be efficient towards the depolymerisation of suberin from birch outer bark. Many other renewable resources are now being explored in our laboratory, aiming the depolymerisation of suberin (*e.g.* potato peels and cantaloupe rind) or cutin (*e.g.* tomato and apple peels). These new biopolyester materials, especially due to their inherent biocompatibility and potential antimicrobial activities, may foster a broad diversity of applications, from the more mundane to rather sophisticated ones. Of course significant work is still required, especially when considering that the chemical and biological

variability amongst species may hamper the film-forming ability of the ensuing extracted biopolyester.

In addition, the partial depolymerised suberin, which is related to *in-situ* suberin, is also being used in our laboratory to investigate fungal degradation mechanisms. Suberised cell walls constitute protective barriers against the pathogenic infections[13,14]; hence the study of its degradation mechanism will certainly be important for the understanding of fungal-plant interactions.

1.1 Janus face *of ionic liquid*

A final comment seems to be appropriate, regarding the selected ionic liquid. Cholinium hexanoate is biocompatible, biodegradable and allow the selective extraction of suberin from its natural sources with high yields. Nevertheless, there are some obstacles that need to be solved to assure its plentiful use.

The combination of the organic cation and anion will determine the degradation temperature of the ionic liquid.[10] Carboxylate anions are good hydrogen bond acceptors (*i.e.* basic), which normally exhibit low stability[10] and thus low decomposition temperatures. Accordingly, during the 4h of extraction at 100 °C about 3 wt% of the ionic liquid is degraded. Moreover, NMR and ESI-MS analysis of the recovered ionic liquid revealed the presence of small amounts of aliphatic contaminants. Although preliminary tests have been made to recover and reuse the ionic liquid at the end of the process, only one cycle is not enough to assure a cost-efficient process. Other important aspects regarding the selected ionic liquid are its melting temperature and viscosity. Cholinium hexanoate melts around 62 °C and shows high viscosity, making the filtration step used to remove the cork insoluble residue extremely time-consuming. At laboratory scale, dimethyl sulfoxide was added at the end of the extraction process to reduce the viscosity of the ionic liquid, thus facilitating the filtration step. Nevertheless, preliminary scale-up tests have showed that the ensuing reaction mixture can be immediately filtrated in a pressurised tank at *ca.* 80 °C. Moreover, the large quantity of water required for suberin precipitation after the filtration may hamper the greenness of this process since the ionic

liquid recovery relies on the evaporation of this antisolvent. Such process represents significant time and energy consumption.

Most of the identified problems are similar to those found in other ionic liquid approaches used towards biomass processing.[10] In this scope ionic liquids have undoubtedly major advantages when compared to common solvents, although improvements are needed to advance from the proof-of-concept to viable applications.

Many challenges could be envisaged here in order to improve the ionic liquid process. For instance, the design of a new ionic liquid that contemplates a low melting temperature, low viscosity and high thermal and chemical stability would be desirable. This would allow simpler recyclability with virtually no losses of solvent. The conceived improvements, however, would need to ensure the main advantage of the overall process, the partial preservation of suberin *in-situ* structure.

2. Acknowledgments

I would like to thank the members of Applied Environmental Mycology Laboratory, involved in suberin and cutin research, namely Rui Ferreira, Marina Guerreiro and Isabel Martins. I'm also very grateful especially to Dina Mestre, Isabel Martins and Celso Martins that will carry on the above mentioned studies.

3. References

1. M. Petkovic, J. L. Ferguson, H. Q. N. Gunaratne, R. Ferreira, M. C. Leitão, K. R. Seddon, L. P. N. Rebelo and C. Silva Pereira, Novel biocompatible cholinium-based ionic liquids—toxicity and biodegradability, *Green Chem.*, 2010, **12**, 643-649.
2. I. Kilpeläinen, H. Xie, A. King, M. Granstrom, S. Heikkinen and D. S. Argyropoulos, Dissolution of wood in ionic liquids, *J. Agr. Food Chem.*, 2007, **55**, 9142-9148.
3. D. A. Fort, R. C. Remsing, R. P. Swatloski, P. Moyna, G. Moyna and R. D. Rogers, Can ionic liquids dissolve wood? Processing and analysis of lignocellulosic materials with 1-*n*-butyl-3-methylimidazolium chloride, *Green Chem.*, 2007, **9**, 63-69.
4. P. Stepnowski, A. C. Składanowski, A. Ludwiczak and E. Łaczyńska, Evaluating the cytotoxicity of ionic liquids using human cell line HeLa, *Hum. Exp. Toxicol.*, 2004, **23**, 513-517.
5. S. Stolte, S. Abdulkarim, J. Arning, A.-K. Blomeyer-Nienstedt, U. Bottin-Weber, M. Matzke, J. Ranke, B. Jastorff and J. Thöming, Primary biodegradation of ionic liquid cations, identification of degradation products of 1-methyl-3-octylimidazolium chloride and electrochemical wastewater treatment of poorly biodegradable compounds, *Green Chem.*, 2008, **10**, 214-224.

6. J. Graça and H. Pereira, Methanolysis of bark suberins: Analysis of glycerol and acid monomers, *Phytochem. Analysis*, 2000, **11**, 45-51.
7. A. Gandini, C. Pascoal Neto and A. J. D. Silvestre, Suberin: A promising renewable resource for novel macromolecular materials, *Prog. Polym. Sci.*, 2006, **31**, 878-892.
8. P. C. R. O. Pinto, A. F. Sousa, A. J. D. Silvestre, C. Pascoal Neto, A. Gandini, C. Eckerman and B. Holmbom, *Quercus suber* and *Betula pendula* outer barks as renewable sources of oleochemicals: A comparative study, *Ind. Crop. Prod.*, 2009, **29**, 126-132.
9. J. Graça and S. Santos, Suberin: A biopolyester of plants' skin, *Macromol. Biosci.*, 2007, **7**, 128-135.
10. A. Brandt, J. Gräsvik, J. P. Hallett and T. Welton, Deconstruction of lignocellulosic biomass with ionic liquids, *Green Chem.*, 2013, **15**, 550-583.
11. P. Aduri, P. V. Uppara, U. Ratnaparkhi and M. Sakhalkar, Hydrolysis and esterification with acid catalysts, *World Pat.*, IN201101585-I3; WO2012164573-A2, 2012.
12. H. Wang, L. Chen, H. Gao, C. Liu and Z. Yu, Preparing biodiesel by using amino acid ionic liquid for catalyzing hydrolysis of castor oil, comprises blending *e.g.* oil, determining glycerol content, allowing to stand to obtain crude biodiesel phase and amino acid ionic liquid phase, *CN Pat.*, CN101974372-A, 2011.
13. K. Ranathunge, L. Schreiber and R. Franke, Suberin research in the genomics era—New interest for an old polymer, *Plant. Sci.*, 2011, **180**, 399-413.
14. P. E. Kolattukudy, Polyesters in higher plants, in: *Biopolyesters*, ed. T. Scheper, W. Babel and A. Steinbuchel, 2001, vol. **71**, pp. 1-49.

“It always seems impossible until it’s done.”
Nelson Mandela

ITQB-UNL | Av. da República, 2780-157 Oeiras, Portugal
Tel (+351) 214 469 100 | Fax (+351) 214 411 277

www.itqb.unl.pt